Modulatory role of vitamin A on the *Candida albicans*-induced immune response in human monocytes

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I TABLE OF CONTENTS

II LIST OF ABBREVIATIONS

III SUMMARY

IV ZUSAMMENFASSUNG

1. INTRODUCTION

1.1 *CANDIDA ALBICANS*

1.1.1 *C. albicans* and its clinical importance

1.1.2 Epidemiology

1.1.3. *C. albicans* pathogenesis

1.1.4 Fungal recognition and host defence against *C. albicans*

1.1.4.1 The innate immune system in *C. albicans* infections

1.1.4.2 The role of monocytes in the immune response against *C. albicans*

1.2 SEPSIS: A DEREGULATION OF THE IMMUNE RESPONSE

1.2.1 Interferon type I and IFNAR axis, an important pathway in the pathogenesis of systemic candidiasis

1.3 NUCLEAR RECEPTORS

1.3.1 Definition and general clinical importance of NRs

1.3.2 Nomenclature of NRs

1.3.3 General structure and mechanism of NRs

1.3.4 General function of NRs

1.3.5 NRs are modulators of the immune response in fungal infections

1.3.5.1 The glucocorticoid receptor (GR) in fungal infection

1.3.5.2 The estrogen receptors (ERs) in fungal infection

1.3.5.3 The peroxisome proliferator-activated receptors (PPARs) in fungal infection

1.3.5.4 The vitamin D receptor (VDR) in fungal infection

1.4 VITAMIN A AND THE INTERACTION WITH ITS NRs: THE RETINOIC ACID RECEPTORS

1.4.1 General importance of vitamin A

1.4.2 Uptake, metabolism and storage of vitamin A

1.4.3 Biological function of vitamin A and its NRs: the RARs

1.4.4 Clinical importance of vitamin A in the immune system

2. HYPOTHESIS/ AIM OF THE THESIS

3. MATERIALS AND METHODS

3.1 MATERIALS: Tables 1- 10

Tab.1 Materials used for cultivation of *C. albicans*

Tab.2 Assessment of cell viability and cell count

Tab.3 Materials used for primary cell isolation

Tab.4 Materials used for cell-culture in stimulation assay

Tab.5 Materials for RNA-isolation and reverse transcription

5.1 Materials for RNA-isolation

5.2 Material for reverse transcription from RNA into cDNA

Tab.6 Materials for semi-quantitative PCR and gel electrophoresis

Tab.7 Materials for real-time quantitative PCR (RT-qPCR)

7.1 Components for RT-qPCR

7.2 Primer of genes used in this study

Tab.8 Cell media

8.1 Medium for monocyte isolation

8.2 Medium for cell culture

8.3 Medium for flow cytometry-analysis

Tab.9 Antibodies and synthetic agonists/ antagonists for stimulation assay

9.1 Antibodies for flow cytometry

9.2 Synthetic agonists/ antagonists

9.2.1 TLR- agonists for the PBMC co-fungal stimulation assay

9.2.2 Retinoic acid receptors stimulation assay

Tab.10 Materials for ELISA

3.2 METHODS
3.2.1 *C. albicans*
3.2.2 Monocyte and PBMC isolation
3.2.2.1 Monocytes stimulation assay
3.2.2.2 PBMC stimulation assay
3.2.3 Preparation of the all-trans retinoic acid
3.2.4 Preparation of RAR-agonists/ -antagonists
3.2.5 Preparation of β-1.3 glucan beads
3.2.6 Preparation of TLR-agonists
3.2.7 RT- PCR and semi-quantitative PCR
3.2.7.1 RNA-Isolation
3.2.7.2 Quantification of purified mRNA
3.2.7.3 Reverse transcription
3.2.7.4 Primer design
3.2.7.5 Semi-quantitative PCR
3.2.7.6 Gel electrophoresis
3.2.7.7 Real-time quantitative PCR
3.2.8 Flow cytometry
3.2.9 Cytokine measurement
3.2.10 Statistical analysis

4. RESULTS
4.1 AtRA is able to modulate the *C. albicans*-induced cytokine production in human monocytes
4.2 AtRA modulates the Dectin-1-mediated cytokine production in human monocytes
4.3 AtRA modulates the expression of relevant PRRs
4.3.1 The expression of Dectin-1 is modulated by atRA
4.3.2 Impact of atRA on the expression of Dectin-1 co-receptors
4.3.3 Early regulation of PRRs by atRA
4.3.4 Short versus long atRA pre-treatment
4.4 AtRA exerts its anti-inflammatory effect in a RAR-dependent manner
4.4.1 RAR expression pattern in human monocytes
4.4.2 Agonist/ antagonist experiments

4.5 Effect of atRA on the interferon type I response against *C. albicans*
4.5.1 AtRA modulates the IFN-β expression upon *C. albicans* and β-1.3 glucan challenge
4.5.2 AtRA modulates the expression of several members of the interferon type I pathway

5. DISCUSSION

6. CONCLUSION

7. BIBLIOGRAPHY / LIST OF REFERENCES

8. SUPPLEMENT
8.1 AFFIDAVIT
8.2 ACKNOWLEDGEMENT

II LIST OF ABBREVIATIONS

AF-1	Activation function region-1
AIDS	Acquired immune deficiency syndrome
AP-1	Activator protein 1, transcription factor
APC	Allophycocyanin
atRA	All-trans retinoic acid
bp	Base pair
C. albicans (UV)	UV-inactivated *C. albicans* yeasts
CARD 9	Caspase recruitment domain family, member 9
cDNA	Single stranded complementary DNA
c-Jun	Transcription factor AP-1-like
CLR	C-type lectin-receptors
Ct	Cycle threshold
CTE	COOH-terminal extension
CRABP	Cellular retinoic acid binding proteins
CO_2	Carbon dioxide
DBD	DNA-binding domain
DBPS	Dulbecco´s phosphate-buffered saline
DCs	Dendritic cells
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DR	Directed repeat
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Gal3	Galectin-3
GM–CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase

HEK 293	Human embryonic kidney cell line
HRE	Hormone response element
HSP	Heat-shock protein
ICU	Intensive care unit
IFN	Interferon
IFN I	Interferon type I
IFN-α	Interferon, alpha
IFN-β	Interferon, beta
IFN-ε	Interferon, epsilon
IFN-ω	Interferon, omega
IFNAR	Interferon-alpha/beta receptor
IL	Interleukin
IRF	Interferon-regulatory factor
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
LXR	Liver x receptor
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NaCl	Natrium chloride
NF-κB	Nuclear factor “kappa-light-chain-enhancer” of activated B-cells
NHBE	Human bronchial epithelial cells
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PE	Phycoerythrin
PICU	Paediatric intensive care unit
PPAR	Peroxisome proliferator-activated receptor
PRAME	Preferentially expressed antigen in melanoma
PRR	Pattern recognition receptor
RAE	Retinol activity equivalent
RBP	Retinol binding protein
Raf-1	Raf-1 proto-oncogene, serine/threonine kinase

RARα	Retinoic acid receptor, alpha
RARβ	Retinoic acid receptor, beta
RARγ	Retinoic acid receptor, gamma
RIP140	Receptor interacting protein 140
RLT	RNeasy lysis buffer
RNA	Ribonucleic acid
RT- qPCR	Real-time quantitative polymerase chain reaction
RXR	Retinoid x receptor
SIRS	Systemic inflammatory response syndrome
dsDNA	Double stranded DNA
ssDNA	Single stranded DNA
STAT	Signal Transducers and Activators of Transcription
TBE	Tris-Borate-EDTA-buffer
TE	Tris-EDTA
TGF-β	Transforming growth factor β
T_H-cells	T-helper cells
THP-1	Human monocytic cell line
TNFα	Tumor necrosis factor alpha
TLR	Toll-like receptor
TR	Thyroid receptor
US	United States
UV	Ultra-violet light
VAD	Vitamin A deficiency
VDR	Vitamin D receptor
YPD	Yeast extract peptone dextrose
$1.25(OH)_2D_3$	Calcitriol, Vitamin D_3, 1α,25-Dihydroxycholecalciferol

Statistics

SEM	Standard error of the mean
ANOVA	Analysis of variance

Units

pg	Picogram
ng	Nanogram

nm	Nanometre
µg	Microgram
µl	Microlitre
µM	Micromole
mg	Milligram
ml	Millilitre
mM	Millimole
cm	Centimetre
g	Gram
J	Joule
l	Litre
min	Minutes
M	Mole
rpm	Rounds per minute
h	Hour
°C	Celsius
V	Volt

III SUMMARY

The scientific background and current state of research:

In the last decades, the frequency of invasive mycoses is on a rise. Among fungi, *C. albicans* is the most common pathogen in bloodstream infection, associated with a high morbidity and mortality rate.

Beside its well-known characteristics in embryogenesis, reproduction and maintenance of the human body tissues, vitamin A and its nuclear receptors (NR1B1, NR1B2 and NR1B3) have shown important immunomodulatory effects in the innate and adaptive immune response. In infectious diseases, treatment with vitamin A has been shown to be host protective in infections of viral, bacterial and protozoan origin. Nevertheless, nothing is known about its impact in fungal infections. Whereas certain nuclear receptors such as PPARs (NR1C1, NR1C2 and NR1C3), GR (NR3C1), ERs (NR3A1 and NR3A2) and the VDR (NR1I1) have already shown to have an influence on the outcome of fungal infections, the potential immunomodulatory role of vitamin A is largely unknown.

Interrogation and targets:

In our present work, we investigate the role of all-trans retinoic acid (atRA), the most active metabolite of vitamin A, on the innate immune response against *C. albicans* in human monocytes.

Methods:

Human monocytes were treated with UV-killed *C. albicans* yeasts.

We analyzed the expression of the cytokines TNFα, IL6, IL12b, IL10 and the PRRs Dectin-1, TLR2 and Gal-3 on transcriptional and post-translational level by real-time qPCR, ELISA and flow cytometry. Furthermore, we analyzed the role of vitamin A on the specific Dectin-1-induced antifungal immune response after stimulating monocytes with the Dectin-1 ligand β-1.3 glucan, a common PAMP of the *C. albicans* cell wall.

Furthermore, we analyzed the impact of vitamin A on the interferon-β expression as well as well-known important receptors and regulatory factors of the interferon type I pathway in *C. albicans* infection.

Results and discussion:

We observed a strong immunomodulatory role of vitamin A, leading to a downregulation of most of the *C. albicans*-induced expression and secretion of the pro-inflammatory cytokines TNFα, IL6, IL12b. Moreover, atRA significantly downregulated the expression of Dectin-1, a pivotal PRR in *C. albicans* infection as well as the Dectin-1-dependent cytokine production.

Both RAR-dependent and RAR-independent mechanisms seem to be involved in the atRA-mediated immune modulation.

Furthermore, our results show an immunomodulatory impact of atRA in the key-players of the interferon type I pathway, well known to have a prominent role in *C. albicans* infections.

Conclusion:

Vitamin A showed an important immunomodulatory impact on the innate immune response in *C. albicans* infection. Our findings open a new direction to elucidate the role of vitamin A on the immune function during invasive fungal infection. It might be a new approach for further new antifungal substances and therapeutic strategies.

IV ZUSAMMENFASSUNG

Wissenschaftlicher Hintergrund und der aktuelle Forschungsstand:

In den letzten Jahrzehnten ist die Anzahl der systemischen Pilzinfektionen dramatisch gestiegen. *C. albicans* repräsentiert den vierthäufigsten nachgewiesenen Erreger in Blutkulturen in an Sepsis erkrankten Erwachsenen. Auf Intensivstationen ist dieser Erreger sogar die dritthäufigste Ursache für Sepsis bei Erwachsenen und Kindern.

In Pilzinfektionen, ist *C. albicans* der am meist vorkommende und nachgewiesene Erreger in Infektionen des Blutkreislaufes. Systemische *C. albicans* Infektionen sind häufig verbunden mit einer hohen Morbiditäts- und Mortalitätsrate.

Vitamin A ist ein wichtiger Baustoff in der Embryogenese, in der Fortpflanzung, in der Zelldifferenzierung und in dem Zellwachstum sowie vielen weiteren biologischen Stoffwechselvorgängen. Neben diesen bedeutungsvollen Fertigkeiten wurden für Vitamin A und seinen nuklearen Rezeptoren (NR1B1, NR1B2, NR1B3) wichtige immunmodulatorischen Fähigkeiten im angeborenen und erworbenen Immunsystem nachgewiesen. Die Therapie mit Vitamin A und seinen Vertretern zeigte in Infektionskrankheiten, die durch Bakterien, Protozoen oder Viren verursacht wurden einen protektiven Effekt. Allerdings gibt es keine Informationen über die Wirkung von Vitamin A in Pilzinfektionen.

Jedoch konnte man in einigen anderen nuklearen Rezeptoren, wie die PPARs (NR1C1, NR1C2 und NR1C3), GR (NR3C1), ERs (NR3A1 und NR3A2) und dem VDR (NR1I1) Einflüsse in der Pathogenität der Pilze nachweisen.

Weiterhin scheint der Interferon Typ I Signalweg, vor allem die Interferon-β Antwort eine wichtige Rolle in der systemischen *C. albicans* Infektion zu spielen. Die Typ I Interferone sind schon lange ein wichtiger Bestandteil in der effektiven Therapie von Infektionen mit Bakterien und Viren. Der Einfluss von Vitamin A in diesem Signalweg ist bisher unbekannt.

Fragestellung und Zielsetzung:

In unserer Arbeit untersuchen wir die Funktion von all-trans Retinsäure (atRA), dem wichtigsten aktiven Metaboliten von Vitamin A, auf das angeboren Immunsystem gegen *C. albicans* in menschlichen Monozyten.

Methodik:

Menschliche Monozyten wurden mit UV-Licht inaktivierten *C. albicans* Hefepilzen behandelt. Die Expression der Zytokine TNFα, IL6, IL12b, IL10 und der Pattern-Recognition-Rezeptoren (PRRs) Dectin-1, TLR2 und Gal-3 wurde auf transkriptioneller und

post-translationaler Ebene mit Hilfe von Real-Time qPCR, ELISA und der Durchflusszytometrie untersucht. Weiterhin haben wir die Rolle von Vitamin A auf die spezifische Dectin-1 vermittelte Immunantwort auf transkriptioneller Ebene untersucht. Hierfür wurden Monozyten mit β-1.3 glucan beschichteten Beads stimuliert, einem spezifischen Ligand von Dectin-1 der in der Zellwand von *C. albicans* zu finden ist. Weiterhin, wurde der Effekt von Vitamin A auf die Expression von Interferon-β in der Infektion mit *C. albicans* erforscht. Es wurde der Einfluss von atRA auf wichtige Rezeptoren und regulierende Faktoren des Interferon Typ I Pfades analysiert. Dies sind Faktoren, deren wichtige Rolle in *C. albicans* Infektionen schon nachgewiesen wurden.

Ergebnisse und Diskussion:

Wir beobachteten eine starke immunmodulatorischen Fähigkeit von Vitamin A auf die Monozyten in der *C. albicans* Infektion. Weiterhin beobachteten wir eine Suppression der Expression und Sekretion der von *C. albicans*-induzierten pro-inflammatorischen Zytokinen TNFα, IL6, IL12b. Weiterhin konnten wir eine signifikante Herabregulierung der Dectin-1 Expression beobachten, einem wichtigen PRR in der *C. albicans* Infektion, sowie auch in der Dectin-1 abhängigen Zytokine Produktion. RAR-abhängige sowie auch RAR-unabhängige Mechanismen scheinen in der atRA-vermittelten Immunmodulation eine Rolle zu spielen. Weiterhin zeigten unsere Ergebnisse einen immunmodulatorischen Effekt von Vitamin A auf die Expression von Interferon-β sowie auf die wichtigsten Faktoren des Interferon Typ I Signalweges.

Schlussfolgerung:

Vitamin A zeigte einen signifikanten einflussreichen immunmodulatorischen Effekt auf die Immunantwort des angeborenen Immunsystems in *C. albicans* Infektionen. Mit Hilfe unserer Ergebnisse könnten neue Wege aufgezeigt werden, um die Rolle von Vitamin A auf die Funktion des Immunsystems während systemischer Pilzinfektionen aufzuklären und zu erläutern. Weiterhin, könnten hiermit neue Angriffspunkte für innovative Therapiestrategien in der Behandlung von systemischen Mykosen entwickelt werden.

1. INTRODUCTION

Modulatory role of vitamin A on the *Candida albicans*-induced immune response in human monocytes

1.1 CANDIDA ALBICANS

1.1.1 *C. albicans* and its clinical importance

Invasive fungal infections are a frequent cause of high mortality and morbidity, especially in immunocompromised and hospitalized adults and children (Brissaud *et al.*, 2012, Gudlaugsson *et al.*, 2003, Kao *et al.*, 1999, Lepak & Andes, 2011, Steinbach, 2010).

Clinical manifestation of *C. albicans* are spreading from mucosal epithelial infection, mostly found in healthy- and moderate immunocompromised patients, to disseminated infection mostly seen in critically ill patients (Gudlaugsson *et al.*, 2003, Martin *et al.*, 2011, Xie *et al.*, 2008).

Among all fungi, *Candida* species, especially *C. albicans* is the most common nosocomial blood stream pathogen. It is capable to cause opportunistic systemic infections, associated with a high risk in sepsis leading to septic shock and multi-organ failure (Arendrup, 2013, Brissaud *et al.*, 2012, Guzman *et al.*, 2011, Leroy *et al.*, 2009, Otto *et al.*, 2011, Xie *et al.*, 2008)

1.1.2 Epidemiology

Over the past decades, the incidence of fungal agents is on a rise, especially among hospital-acquired opportunistic infections (Pfaller & Diekema, 2010, Tortorano *et al.*, 2006).

Invasive fungal infections are an increasing feared condition in critically ill patients in the ICU and PICU (Bajwa & Kulshrestha, 2013, Brissaud *et al.*, 2012, Delaloye & Calandra, 2014, Matthaiou *et al.*, 2015). Among critically ill patients, *C. albicans* is one of the most prevalent invasive fungal agents associated with a high morbidity and mortality rate (Bloos *et al.*, 2013, Majer *et al.*, 2012, Netea *et al.*, 2006). Candideamia represents the 4th most common cause in hospital acquired bloodstream infections (Bajwa & Kulshrestha, 2013,

Lewis, 2009). Moreover, it ascended to the 3rd most common cause in bloodstream infections in adults in intensive care units and in health-care associated blood stream infection among children and neonates (Brissaud *et al.*, 2012, Lewis, 2009).

Despite the availability of novel classes of antifungal agents, in patients with impaired immunity, nosocomial candideamia is still associated with a high mortality ranging from 30% to 50% in US and Europe (Castelli *et al.*, 2014, Guzman *et al.*, 2011, Tortorano *et al.*, 2006, van de Veerdonk *et al.*, 2010). In fact, Lepak *et al.* reported an attributable mortality rate of up to 75 % caused by *Candida spp.* (Lepak & Andes, 2011).

Although the new antifungal agents show lower toxic side effects, early identification and subsequent treatment in the clinical daily life is still a great challenge (Badiee & Hashemizadeh, 2014, Castelli *et al.*, 2014).

In the last years, it has become clear that the host immune response plays a crucial role in the type and severity of *Candida* infection. If a patient appears in a healthy state, colonization by *C. albicans* and the host defence is balanced (Cantorna & Balish, 1990). *C. albicans* is a common part of the flora of the skin, gastrointestinal and genitourinary tract (Kumamoto, 2011). If an imbalance between the host defence and colonization of *C. albicans* in non-immunocompromised patient occurs, mucosal membrane infection like oropharyngeal or vaginal infection are quiet commonly seen. Nearly 75 % of all women and 70 % of the general population experience an overgrowth of *Candida* at some point in their lifetime (Mascellino, 2013, van de Veerdonk *et al.*, 2010). Several factors of the host have been identified to increase the risk of invasive *Candida* infection (Achkar & Fries, 2010, Anwar *et al.*, 2012, Brissaud *et al.*, 2012, Guzman *et al.*, 2011, Muskett *et al.*, 2011, Pallavan *et al.*, 2014, Park, 2005, Relloso *et al.*, 2012, Saiman *et al.*, 2000, Sano *et al.*, 2014, van de Veerdonk *et al.*, 2010). The most important risk factors are low immune response or immune suppression induced either by neutropenia for more than 10-15 days (Brissaud *et al.*, 2012), chemotherapy (Mech *et al.*, 2014, Sano *et al.*, 2014, Zipfel *et al.*, 2011), AIDS (Anwar *et al.*, 2012, Sano *et al.*, 2014), transplantation (Brissaud *et al.*, 2012, Mech *et al.*, 2014, Zipfel *et al.*, 2011), diabetes mellitus (Guzman *et al.*, 2011, Pallavan *et al.*, 2014) and others (Achkar & Fries, 2010, Anwar *et al.*, 2012, Brissaud *et al.*, 2012, Guzman *et al.*, 2011, Muskett *et al.*,

2011, Pallavan *et al.*, 2014, Park, 2005, Relloso *et al.*, 2012, Saiman *et al.*, 2000, Sano *et al.*, 2014, van de Veerdonk *et al.*, 2010, Zipfel *et al.*, 2011). In the state of low immune response, *C. albicans* turns into an opportunistic pathogen, which is able to translocate in the body by invading the epithelial barrier (Felk *et al.*, 2002). Through haematogenous spreading invasive focal infection like pyelonephritis, meningitis, endocarditis, pneumonia, peritonitis or moreover systemic distribution leading into life-threatening infections might occur (Pallavan *et al.*, 2014, Saiman *et al.*, 2000, Sano *et al.*, 2014). Other risk factors like hormonal imbalance generated by the intake of estradiol, long-term glucocorticoid therapy or diabetes mellitus support the susceptibility to invasive fungal infections (Relloso *et al.*, 2012). Furthermore, Vitamin deficiency is reported as another risk factor (Sano *et al.*, 2014). Additionally, one of the main risks for invasive fungal infection is the period during a hospital stay (Guzman *et al.*, 2011, Muskett *et al.*, 2011).

Hospital-acquired infections are the most common invasive fungal infections, especially during the stay in intensive care unit. 40% of invasive C*andida* infections occur in critical care units (Muskett *et al.*, 2011). Indwelling catheters, renal replacement therapy, artificial mechanical ventilation, large surgeries, total parenteral nutrition, and long-term broad-spectrum antibiotic therapy are important risk factors of a life-threatening fungal disease (Anwar *et al.*, 2012, Brissaud *et al.*, 2012, Guzman *et al.*, 2011, Lamagni *et al.*, 2001, Muskett *et al.*, 2011, Pallavan *et al.*, 2014, Park, 2005, Pfaller & Diekema, 2010, Relloso *et al.*, 2012, Saiman *et al.*, 2000, Sano *et al.*, 2014). Nonetheless, the number of invasive *Candida* infections has dramatically increased, not only observed in immune compromised patients or in critically ill patients with severe serious underlying disease, but rather increasingly seen in outpatient and non-hospital settings (Kao *et al.*, 1999, Lamagni *et al.*, 2001, Pfaller & Diekema, 2010).

1.1.3 *C. albicans* pathogenesis

C. albicans is a yeast fungus, able to change reversibly its morphological stages from yeast to pseudo hyphae into budded hyphae according to its need (Jacobsen *et al.*, 2012). Through the hyphal form, *C. albicans* is able to invade the host tissue and further escape out of the host cells after its internalization (Felk *et al.*, 2002, Filler & Sheppard, 2006, Jacobsen *et al.*, 2012, Nobile *et al.*, 2012, Toenjes

et al., 2005). Inside the human body a morphological change into the yeast form allows *C. albicans* to circulate in the blood for haematogenous dissemination (Brand, 2012, Nobile *et al.*, 2012). Both yeast and hyphal cells of *C. albicans* are found in infected host tissues (Jacobsen *et al.*, 2012, Spellberg & Edwards, 2002). Several influential factors have been proven to induce a morphological change: high temperature, pH above 6.5, anaerobic environment and nutrient starvation (Ernst, 2000, Gow *et al.*, 2012). Further virulence factors of *C. albicans* are its ability to form biofilms and to secret hydrolytic enzymes. Moreover, its broad genetic diversity within the species of around 6000 predicted genes and its ability to stimulate the anti-inflammatory immune response of the host are further important virulence factors of *C. albicans* (Brand, 2012, Cannom *et al.*, 2002, Nobile *et al.*, 2012, Perez-Nadales *et al.*, 2014, Spellberg & Edwards, 2002). Although virulence factors and host risk factors, which enable an overgrowth of *C. albicans* have been identified in last few years, clear mechanism that endeavour *C. albicans* from a commensal to a severe fungal pathogen are still less clear. Although, novel antifungal agents are already present, the fungal broad-spectrum genetic diversity leads into recurring resistance. Furthermore, their toxic side effects and drug-drug interactions lead these agents to still restricted usage. These mentioned challenges and the late diagnosis by the difficulties to recognize afflicted patient tissues still upholds *C. albicans* as a remaining severely dangerous pathogen (Castelli *et al.*, 2014, Perez-Nadales *et al.*, 2014)

1.1.4 Fungal recognition and host defence against *C. albicans*

The first and essential path in the host immune defence is the capability of the host to recognize invaders by specific pattern recognition receptors (PRRs) on the surface or at the lysosome of several innate immune cells (Akira *et al.*, 2006).

1.1.4.1 The innate immune system in *C. albicans* infections

The innate immune system plays a crucial role in the first line of defence by recognizing invaders, responding by nonspecific defence mechanisms and activation of the specific adaptive immune system (Kumar & Sharma, 2010). Anatomical barriers like the skin, the complement system, various immune cells and their secreted mediators are all part of the innate immune system (Kumar &

Sharma, 2010).

PRRs are ubiquitously expressed receptors on the surface or in the endosomal compartment of several immune cells and assume a critical part of the innate immune system (Akira *et al.*, 2006, Elinav *et al.*, 2011, Loo & Gale, 2011). Four classes of PRRs have been described until now: Toll-like - (TLRs), C-type lectin - (CLRs), Nod-like - (NLRs) and RIG-I-like receptors (RLRs) (Akira *et al.*, 2006, Elinav *et al.*, 2011, Loo & Gale, 2011). PRRs enable the recognition of unique pathogen associated molecular patterns (PAMPs). PAMPs are conserved microbial structures, which are not present in the host and share a number of different recognizable biochemical features that alert the organism to intruding pathogens (Akira *et al.*, 2006, Bianchi, 2007). Well-known PAMPs include lipopolysaccharides (LPS) of gram-negative bacteria, β-glucans and mannans of *C. albicans* and different types of nucleic acids of pathogens of bacterial, viral or fungal origin (Akira *et al.*, 2006). Activated PRRs initiate an effective cellular response by inducing the innate and adaptive immune defence either directly through the secretion of pro-inflammatory cytokines, chemokines or other mediators, or through the activation of specific signalling pathways (Akira *et al.*, 2006, Akira, 2009, Elinav *et al.*, 2011, Hontelez *et al.*, 2012, Loo & Gale, 2011). Most PRR signalling pathways in fungal infections lead to the activation of the NF-κB protein complex, unique regulators of the gene transcription (Hardison & Brown, 2012, Kawai & Akira, 2006). The NF-κB protein complex is capable to induce or amplify the gene expression of several genes in immune cells, leading to an increased production and release of specific necessary mediators to eliminate pathogens (Hardison & Brown, 2012, Kawai & Akira, 2006). Nevertheless, in recent years it has become clear, that not only one PRR but moreover a cross talk of several PRRs trigger a complex intercellular signalling cascade leading into an efficient innate and adaptive immune response (Hardison & Brown, 2012, Hontelez *et al.*, 2012).

The fungal cell wall of *C. albicans* comprises two layers of carbohydrates and proteins. These contents represent PAMPs particularly for the CLRs and TLRs (Hontelez *et al.*, 2012). Activated by these PAMPs, several of these PRRs enable the release of pro-inflammatory chemokines and cytokines like TNFα, IL6, IL12b and type I IFN (Huppler *et al.*, 2012, Kim *et al.*, 2005, Majer *et al.*, 2012, Navarathna *et al.*, 2007, Netea *et al.*, 2003, Romani *et al.*, 1996, Steinshamn &

Waage, 1992).

N-and O-linked mannose polymers predominately compose the outer layer of the *C. albicans* cell wall to build up glycoproteins (Gow *et al.*, 2012). The inner layer of the fungal cell wall contains the skeletal polysaccharide chitin and β-1.3 glucan, which confer strength and cell shape, and the more flexible β-1.6 glucan (Gow *et al.*, 2012).

The most important PRR in *C. albicans* infection is the CLR Dectin-1. It recognizes β-1.3 glucan and is described as the key activator to a successful antifungal immune defence (Brown *et al.*, 2002, Wheeler & Fink, 2006).

Furthermore, human immune cells are able to recognize the different mannans of the outer layer of the fungal cell wall by either members of the lectin family: the Galectin-3 receptor (Gal3) and the mannose receptor or by the TLRs: TLR2 and TLR4 (Gow *et al.*, 2012). Whereas both TLRs and the mannose receptor are exclusively found on the surface of several immune cells, Gal-3 is found in different compartments of the human immune cells, in the cytoplasm, membrane or in the nucleus (Akira, 2009, Biondo *et al.*, 2012, Hardison & Brown, 2012, Netea & Maródi, 2010, Vasta, 2012). Gal-3 is described to be an important pro-inflammatory enhancer involved in *C. albicans* elimination (Akira, 2009, Biondo *et al.*, 2012, Hardison & Brown, 2012, Netea & Maródi, 2010, Vasta, 2012).

In humans, Dectin-1, encoded by the CLEC7A gene is expressed in several immune cells of the innate immune system like monocytes, macrophages, dendritic cells and neutrophils. It effectively activates the antifungal host defence by triggering the phagocytosis and the production of pro-inflammatory cytokines leading to further activation of the innate and adaptive immune system through signalling pathways (Brown, 2006, Esteban *et al.*, 2011, Gow *et al.*, 2012). It mainly realises its antifungal effect by the utilisation of the NF-κB protein complex, activated by either the Raf-1 or CARD 9 signalling pathway (Gow *et al.*, 2012, Hardison & Brown, 2012, Lionakis & Netea, 2013).

Dectin-1 is able to recognize β-1.3 glucan at the inner layer of the fungal cell wall in a yeast or budded stage of *C. albicans*, as well as after cell separation. In a hyphal form, the recognition of the β-1.3 glucan in *C. albicans* cell wall is blocked by chitin/mannan (Gantner *et al.*, 2005, Gow *et al.*, 2012, Netea *et al.*, 2008). Nevertheless, collaborations of certain CLRs, TLRs and enhancers like Gal-3 lead to an optimal antifungal response (Gow *et al.*, 2012, Hardison &

Brown, 2012). Especially the crosstalk between Dectin-1, TLR2 and Gal-3 seems to be important for an optimal antifungal host response (Esteban *et al.*, 2011, Jouault *et al.*, 2006). In addition to these main surface PRRs, the intracellular receptors TLR7 and TLR9, located in the lysosome compartment, have been recently reported to play an important role in the modulation of the antifungal response in *C. albicans* infection by recognizing the fungal nucleic acids (Biondo *et al.*, 2012, Biondo *et al.*, 2011, He *et al.*, 2013, Kasperkovitz *et al.*, 2011). Like all other mentioned TLRs, they exert their effect through the activation of the adapter protein MyD88 leading to a modulation of the gene expression by the transcription factor NF-κB (Kawai & Akira, 2006, Kumagai & Akira, 2010, Takeuchi & Akira, 2010).

1.1.4.2 **The role of monocytes in the immune response against *C. albicans***

Monocytes derive from the bone marrow and circulate for 3 to 5 days in the blood. In the blood, monocytes exert multiple functions: patrolling, initiating inflammation, migrating to various tissues of the host, especially in an activated stimulated state and exerting their repairing functions. Furthermore, monocytes are important for maintaining the homeostasis of the immune system (Geissmann *et al.*, 2010, Italiani & Boraschi, 2014, Saeed *et al.*, 2014, Ziegler-Heitbrock *et al.*, 2010). Moreover, these immune cells represent a special linkage between the innate and adaptive immune system not only due to their ability to develop into either DCs or macrophages depending on environmental cytokines and other mediators (Geissmann *et al.*, 2010, Italiani & Boraschi, 2014, Serbina *et al.*, 2008, Ziegler-Heitbrock *et al.*, 2010). In invasive *C. albicans* infections, monocytes represent one of the most important elements in the early antifungal defence (Kim *et al.*, 2005, Quintin *et al.*, 2012, Torosantucci *et al.*, 2004, van de Veerdonk *et al.*, 2009). They are equipped with several PRRs on their cell surface, especially the PRRs Dectin-1, TLR2 and Gal-3 (Bonfim *et al.*, 2009, Liu *et al.*, 1995). Monocytes produce an intricate pattern of cytokines and chemokines that enhance phagocytosis, microbicidal activity and chemotaxis, as well as the activation of T-cells through antigen processing and presentation (Geissmann *et al.*, 2010, Kim *et al.*, 2005, Quintin *et al.*, 2012). Recently, Quintin *et al*. reported a further protective capability of monocytes against a re-infection with *C. albicans*, requiring the Dectin-1 PRR (Quintin *et al.*, 2012).

1.2 SEPSIS: A DEREGULATION OF THE IMMUNE RESPONSE

The adequate amount of released pro-inflammatory cytokines in the early phase of infection determines the further progress in either elimination of the pathogenic invader or development into sepsis (Navarathna *et al.*, 2007, Netea *et al.*, 2003, Romani *et al.*, 1996, Steinshamn & Waage, 1992). A low concentration of cytokines in the early phase of infection leads into an ineffective elimination of the pathogen, whereas an excessive increase in pro-inflammatory mediators in local tissues might result in a cytokine storm, and lead to a massive systemic pro-inflammatory response, the SIRS (Comstedt *et al.*, 2009, Netea *et al.*, 2003). Sepsis, a frightening severe SIRS condition caused by a proven or suspected pathogen is able to lead rapidly into a life-threatening septic shock with cardiovascular impairment, unspecific apoptosis and lastly into MODS, a multiple organic dysfunction syndrome (1992, Bone, 1996, Comstedt *et al.*, 2009, Dellinger *et al.*, 2013, Guzman *et al.*, 2011, Netea *et al.*, 2003, van der Zee, 2010). Candidemia is an increasing source in the development of sepsis, septic shock and multiple organ failure (Guzman *et al.*, 2011, Majer *et al.*, 2012, Martin *et al.*, 2003, Spellberg & Edwards, 2002).

1.2.1 Interferon Type I and IFNAR axis, an important pathway in the pathogenesis of systemic candidiasis

Beside the already mentioned anti-*candida* host defence mechanisms, another pathway has recently been reported to play a relevant role to diminish invasive *C. albicans* infection*:* the interferon type I pathway (IFN I) (Biondo *et al.*, 2011, Majer *et al.*, 2012, Perry *et al.*, 2005, Smeekens *et al.*, 2013).

The IFN I pathway is already known to play a pivotal role in the immune response in viral or bacterial infections (Perry *et al.*, 2005).

In invasive *Candida* infections, interferons are important mediators in the host defence by promoting a pro-inflammatory response of the innate immune system (Majer *et al.*, 2012). Nevertheless, exaggerated activation of inflammatory monocytes and neutrophils by deregulated interferon levels might lead into an uncontrolled hyper-inflammatory response critical to cause host damage and sepsis-related pathology (Majer *et al.*, 2012, Malireddi & Kanneganti, 2013).

The interferon family contains three main classes, classified on the bases of their structure and their use of specific receptors, interferon type I-III (Cho & Kelsall,

2014, Makela *et al.*, 2011, Malireddi & Kanneganti, 2013, Platanias, 2005). In humans, the IFN I include IFN-β encoded by one gene (IFNB1), several subtypes of IFN-α encoded by 14 genes, and others like IFN-ω and IFN-ϵ. Both IFN-β and IFN-α, are reported to be important in invasive fungal infections (Malireddi & Kanneganti, 2013, Platanias, 2005). In systemic *C. albicans* infection, the innate immune system secretes beside several cytokines (mentioned above) interferon-α and interferon-β after the recognition of fungal PAMPs. For the release of interferon-α or interferon-β, fungal PAMPs will either be detected on the surface of immune cells by several PRRs like Dectin-1 or by endosomal located TLRs: TLR7 and TLR9 after the phagocytosis of this fungal invader (Bourgeois *et al.*, 2011, Majer *et al.*, 2012, Makela *et al.*, 2011, Malireddi & Kanneganti, 2013, Platanias, 2005). Especially these TLRs, TLR7 and TLR9, which recognize fungal DNA/RNA, have been shown to be crucial in the induction of the IFN I antifungal response (Biondo *et al.*, 2011). In invasive *Candida* infection, INF-β is reported to be among the most important cytokines functioning as a controller to recruit and activate inflammatory monocytes and neutrophils (Majer *et al.*, 2012). The secreted IFN I binds to a common heterodimeric cell surface interferon α/β receptor (IFNAR), comprised by two chains (IFNAR1 and IFNAR2) and associated with the tyrosine kinases Jak1 and Tyk2 (Malireddi & Kanneganti, 2013). After inducing IFNAR1/2, associated JAK1/Tyk2, tyrosine kinases will recruit and activate the signal transducer and activator of transcription (STAT) by phosphorylation (Bourgeois *et al.*, 2011, Majer *et al.*, 2012, Makela *et al.*, 2011, Malireddi & Kanneganti, 2013, Platanias, 2005, Smeekens *et al.*, 2013). Furthermore, the interferon regulatory factors (IRFs) and the interferon-stimulated genes (ISGs) will be activated leading into an IFN I-mediated paracrine/-autocrine loop (Bourgeois *et al.*, 2011, Majer *et al.*, 2012, Makela *et al.*, 2011, Malireddi & Kanneganti, 2013, Platanias, 2005, Smeekens *et al.*, 2013)

In *C. albicans* infections, the homo-dimerization of STAT1 or hetero-dimerization of STAT1, STAT 2 and the IRF1,-3,-5,-7 or IRF-9 turned out to be the most important parts in the regulation of the IFN I-mediated anti-*C. albicans* host response (Biondo *et al.*, 2011, Bourgeois *et al.*, 2011, Malireddi & Kanneganti, 2013, Smeekens *et al.*, 2013).

Fig. 1: IFN I pathway in monocytes challenged with *C. albicans.*

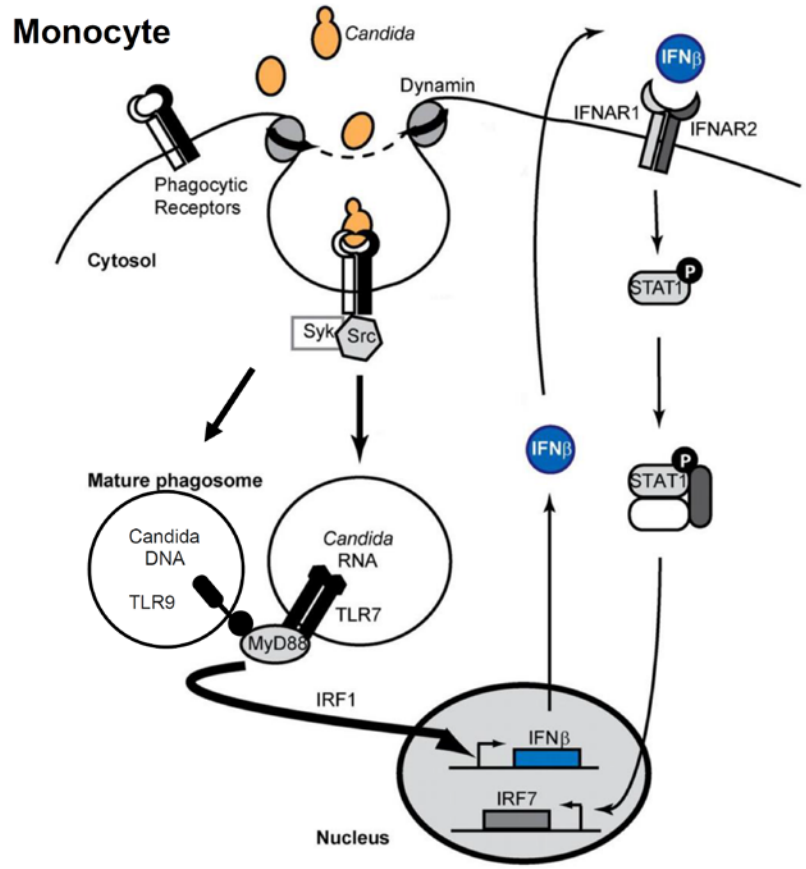

Fig. 1: Modified Illustration by Bourgeios *et al.* 2011 shows the IFN I pathway in monocytes challenged with *C. albicans*. The IFN-β expression will be stimulated after the internalization of *C. albicans* and the recognition of its fungal nucleic acids by the endosomal located TLR7 and TLR9.

In a recent study, IFNAR-deficient mice seem to be protected against fungal sepsis related mortality by lacking to respond to IFN-β immune stimulation (Majer *et al.*, 2012). Interestingly, in this study the addition of the nuclear receptor (NR) PPARγ seemed to modulate and deflate the hyper-inflammatory immune response leading into improved survival (Majer *et al.*, 2012).

1.3 NUCLEAR RECEPTORS

1.3.1 Definition and general clinical importance of NRs

NRs are a large superfamily of about 48 members of ligand- or signal-activated

transcription factors (Germain *et al.*, 2006, Jin & Li, 2010) . They play diverse roles in the homeostasis of cell differentiation, development, proliferation, metabolism and regeneration (1999, Ali *et al.*, 2015, Calkin & Tontonoz, 2012, Saijo *et al.*, 2013). Ligands of NRs include lipophilic endocrine hormones like steroid hormones, thyroid hormones and vitamins like vitamin D_3 or the retinoids, as well as fatty acids, cholesterol and several other mediators. Still not all have been identified so far (Aranda & Pascual, 2001).

Increasing numbers of nuclear hormone receptors are reported to be involved in a large number of pathologies like cancer, cardiovascular disease, inflammation, reproductive abnormalities and metabolic disorders (Aranda & Pascual, 2001, Cheng, 2005, Committee, 1999, Ding *et al.*, 2015, Kleiman *et al.*, 2012a, Lavall *et al.*, 2014b, Matsuda *et al.*, 2013, Staels, 2010, Sutherland *et al.*, 2010, Wetendorf & DeMayo, 2014). Accordingly, NRs have become important therapeutic targets to develop new strategies of treatment, already improving the outcome of different pathologies (1999, Aranda & Pascual, 2001, Cheng, 2005, Ding *et al.*, 2015, Kleiman *et al.*, 2012a, Lavall *et al.*, 2014a, Luo *et al.*, 2014, Matsuda *et al.*, 2013, Staels, 2010, Sutherland *et al.*, 2010, Wetendorf & DeMayo, 2014).

1.3.2 **Nomenclature of NRs**

The nomenclature of NRs is classified by the International Committee of Pharmacology, Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) in a logical numbering system and receptor code like RARα (NR1B1) (Germain *et al.*, 2006). In each manuscript dealing with NRs, it is recommended that the NRs are identified by the official name (code) at least once in the summary and in the introduction (Germain *et al.*, 2006).

Furthermore, NRs can be classified in three groups by their presence and knowledge of its ligands as classic receptors, adopted orphan receptors and orphan receptors (Germain *et al.*, 2006, Jin & Li, 2010). Classic receptors are receptors regulated by already well studied endocrine ligands such as retinoic acids, vitamin D_3, thyroid hormones and steroid hormones (Jin & Li, 2010). Adopted orphan receptors such as PPARs, LXRs, RXRs and others are receptors, for which either synthetic or natural ligands have been identified in recent years (Germain *et al.*, 2006, Jin & Li, 2010, Mangelsdorf *et al.*, 1995b). Orphan

receptors are a class of NRs, for which regulatory ligands still remain unknown or may not exist (Germain *et al.*, 2006).

Additionally, the members of the nuclear hormone receptor superfamily can be classified in several subgroups based on their interaction with the DNA: classified either by their different mechanisms or by their homologues structures (1999, Germain *et al.*, 2006, Jin & Li, 2010, Laudet, 1997, Mangelsdorf *et al.*, 1995a, Olefsky, 2001).

Earlier, four different groups of NRs were identified by Mangelsdorf *et al.* based on their characteristic mechanism (Mangelsdorf *et al.*, 1995a). Nevertheless, one century later, Germain *et al.* classified six different groups of NRs, dividing the 4th group of NRs, the class of orphan receptors into group IV-VI (Germain *et al.*, 2006).

The type I group of NRs unifies twenty members of the thyroid hormone receptor like group combining the thyroid hormone receptors (TRs), retinoic acid receptors (RARs), peroxisome proliferator-activated receptors (PPARs), vitamin D-like receptor (VDR), pregnane x receptor (PXR) , liver x receptor (LXR) as well as several other orphan receptors (Germain *et al.*, 2006).

The type II group of NRs contains the retinoid-x receptor like members: retinoid-x receptor (RXR), the testicular receptors (TRs), the hepatocyte nuclear factor 4 (HNF-4) as well as three orphan receptors (Aranda & Pascual, 2001, Germain *et al.*, 2006).

The type III group of NRs, the estrogen receptor-like group, comprises the estrogen receptors (ERs), estrogen related receptors (ERRs) and the well-known 3-ketosteroid receptors: the mineralocorticoid receptor (MR), the glucocorticoid receptor (GR), the progesterone receptor (PR) and the androgen receptor (AR) (Germain *et al.*, 2006).

The type IV–VI groups of NRs comprise beside the oxysterol mediated Steroidogenic factor 1(SF-1), Fushi Tarazu factor 1 (FTZ-F1) several orphan receptors which are increasingly apparent in the human body, especially for medical therapy (Han *et al.*, 2013, Kohler *et al.*, 2008). However less is known so far about these six members (Aranda & Pascual, 2001).

Furthermore, NRs can be classified into four classes according to their ligand binding, DNA binding, and dimerization properties: steroid receptors, heterodimers, homodimeric orphan receptors, and monomeric orphan receptors

(Mangelsdorf et al., 1995b). Homo- and heterodimeric NRs bind with each partner to their specific response elements sequences on target genes (Olefsky, 2001). According to the class of NRs, the NRs of class I-III bind specific DNA sequences of the target gene as homo- or hetero dimeric partners , whereas NRs of class IV bind specific DNA sequences as monomers (Olefsky, 2001). Heterodimeric NRs mainly enter a heterodimeric partnership with the common retinoid x receptor (Germain *et al.*, 2006). Heterodimer forming partnership with members of the RXR-family can be classified into two groups depending on their functionality as heterodimers: the permissive and non-permissive RXR containing heterodimers (Aranda & Pascual, 2001, Pawlak *et al.*, 2012). Permissive RXR-partners like PPARs and LXRs can be activated either by their own ligands or by RXR-agonists (Aranda & Pascual, 2001, Pawlak *et al.*, 2012). Non-permissive partners like RARs, VDR and TRs cannot be activated by RXR-agonists and require the ligands of their own to be activated (Aranda & Pascual, 2001, Pawlak *et al.*, 2012).

1.3.3 General structure and mechanism of NRs

All nuclear receptors are comprised by a main basic structure, composed of five to six domains from A to F, from the N-terminal to the C-terminal end on the basis of regions of conserved sequence and function (Fig. 2) (Aranda & Pascual, 2001, Germain *et al.*, 2006, Jin & Li, 2010). A hypervariable N-terminus (A/B-domain) linked by a conserved C–region; the DNA-binding domain (DBD); a linking region called the D-domain followed by the conserved ligand-binding domain (LBD), the E/F-region (Jin & Li, 2010).

The N-terminus, a hypervariable NH_2-region also called A/B domain, frequently contains an activation function region 1 (AF-1), capable of activating the gene transcription either in a ligand-dependent or -independent manner (Aranda & Pascual, 2001, Khan & Vanden Heuvel, 2003). This A/B region and the AF-1 region is assumed to be responsible for cell type-specific, species-specific and

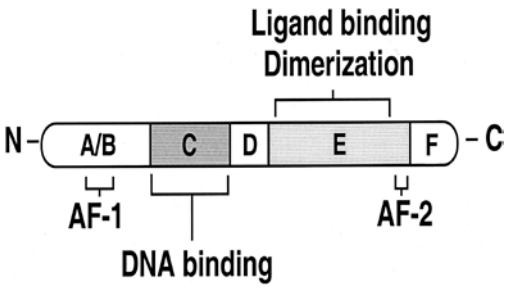

Fig. 2: Basic structure of NRs
by *Aranda & Pascual 2001*

receptor-specific activities. It facilitates "promotor context-dependent properties" (Aranda & Pascual, 2001, Khan & Vanden Heuvel, 2003). It is the target of phosphorylation due to several signalling pathways, like phosphorylation by the cyclin-dependent kinase in the RARs or by the MAPK in the ERs (Aranda & Pascual, 2001, Khan & Vanden Heuvel, 2003). The AF-1 region is variable in size and sequence, and responsible for the different properties of the members of the nuclear receptor family (TR vs. GR) and between their subtypes/ isoforms (RARα and RARβ) (Khan & Vanden Heuvel, 2003). Beside the AF-1 region, another transcriptional activated function region is present in the NRs, the AF-2 region, located near the C-terminus at the LBD. Nevertheless, this AF-2 region is strictly ligand- dependent and conserved among members of the NRs (Aranda & Pascual, 2001). The highly conserved DBD located in the C-region of the NR enables the NR to recognize specific target sequences in the promotor region, the hormone response element (HRE) and to bind with high affinity to the DNA (Aranda & Pascual, 2001, Germain *et al.*, 2006). The promotor region is a specific region of DNA sequences that initiates transcription of a particular target gene. It contains the HRE where NRs bind highly specific to a short nucleotide sequence of a target gene (Aranda & Pascual, 2001, Khan & Vanden Heuvel, 2003). HRE are bipartite elements, composed of one or two hexameric core half-sited motifs. These nucleotide sequences form palindromes (Pal), direct (DR), everted (ER) or inverted repeats (IR) separated by a short spacer of varying length and sequence (Germain *et al.*, 2006, Pawlak *et al.*, 2012). The DBD contains two zinc fingers, a COOH-terminal extension (CTE) and three helices (Aranda & Pascual, 2001, Khan & Vanden Heuvel, 2003). The first zinc finger contains the proximal or P-box region, an α-helix that is responsible for high-affinity recognition of the core half site of the response element (Germain *et al.*, 2006). The second zinc finger contains the distal or D-box, an α-helix which lies perpendicular to the p-box helix and mediates the receptor dimerization (Germain *et al.*, 2006). The CTE contains the third helix and is important for monomeric DNA binding (Aranda & Pascual, 2001).

The hinge region or D-region, a linking region between the DBD and the LBD is a less conserved structure in the NRs and allows rotation of the DBD (Aranda & Pascual, 2001).

With the help of the LBD or E/F region, the NR is able to recognize and bind one, or several ligands (Aranda & Pascual, 2001). Furthermore, this region enables the NR to mediate homo- and heterodimerization, interaction with heat-shock proteins (HSPs), ligand-dependent transcriptional activity and in some cases hormone reversible transcriptional repression (Aranda & Pascual, 2001, Khan & Vanden Heuvel, 2003). The sequence of the LBD differs between NRs as not all recognize the same ligands. Each ligand binds mainly to a ligand-binding pocket, a hydrophobic region surrounded by 10 to 13 α-helices (Aranda & Pascual, 2001, Khan & Vanden Heuvel, 2003).

1.3.4 General mechanism and function of NRs

NRs are ligand or signal activated transcription factors that control transcription by several mechanisms, including both activation and repression activities (Germain *et al.*, 2006). In a classical model, NRs are located in an inactive form either bound to chaperones like HSPs in the cytosol or in the nucleus until the lipophilic ligand enters the cellular or nuclear membrane (McEwan & Kumar, 2015). The presence of the ligand induces a receptor-ligand interaction by binding to the LBD, promoting a conformational change and leading to the dissociation of the HSPs (McEwan & Kumar, 2015). Next the activated receptor-ligand complex in the cytosol can translocate in the nucleus (McEwan & Kumar, 2015). Inside the nucleus, the essential step in the NR-mediated transcriptional activity is the interaction with the specific DNA sequence, the HRE (Germain *et al.*, 2006). NRs, bind according to their class either as homo- , heterodimers (class I-III) or as monomers (class IV) to the HRE (Olefsky, 2001). HREs are often present as two half-site motifs formed as palindromes (head to head), direct repeats (DR) or inverted palindromes (IR) (tail to tail) (Laudet, 1997). Members of the class I NRs, the steroid receptors, mainly bind as homodimers to palindromic sequences composed of six base pairs (bp) arranged as inverted DNA repeats spaced by three bp (IR3)(Aranda & Pascual, 2001, Laudet, 1997, Neill Ph.D., 2006, Pawlak *et al.*, 2012). Furthermore, several non-steroidal NRs can bind their HRE as homodimers, but binding to their HRE as heterodimers increases the efficiency of the DNA binding and their transcriptional activity (Aranda & Pascual, 2001). Most of these NRs form a partnership with members of the RXR-family (Germain *et al.*, 2006). Heterodimers of the class II – III

recognize their HRE in half-core motifs mainly arranged as DRs, but they can also recognize IRs or ERs (Claessens & Gewirth, 2004, Pawlak *et al.*, 2012). The spacing between the DR dictates the type of heterodimer recognizing it, a DR separated by five nucleotides (DR5) will often be recognized by RXR:RAR partnership whereas RXR:TR heterodimer recognize DR4 (Laudet, 1997). Orphan receptors bind as monomers with high affinity to the DNA, utilizing the CTE to sequence (Aranda & Pascual, 2001). After the NR binds to the HRE according to its class, the ligand- nuclear receptor complex is capable to activate the gene transcription by recruiting specific co-activator complexes (Aranda & Pascual, 2001, Saijo *et al.*, 2013). Specific coactivator complexes include the histone acetyltransferase (HAT), chromatin remodelling and mediator complexes and general transcription machineries formed by the RNA-polymerase II and general transcription factors (Germain *et al.*, 2006, Sladek, 2011). Nevertheless, the allosteric effect of the ligand and the HRE on NRs seems to determine which co-activator complexes are recruited and what kind of activities the complex will exert (Claessens & Gewirth, 2004).

Fig. 3: NRs modulate the gene expression by recruiting either co-activator or co-repressor complexes

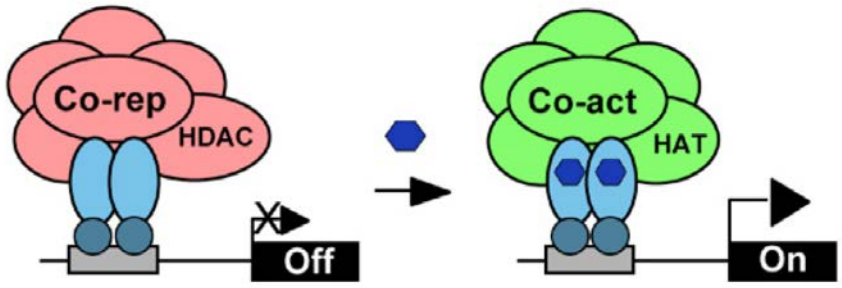

Fig. 3: The figure of Sladek, 2011 shows the NR binding as homo – or heterodimer to HREs, recruiting either co-activator or co-repressor complexes leading to the activation or repression of gene transcription.

Furthermore, NRs are also able to abrogate the transcription by the use of several transrepressor proteins, such as the nuclear receptor corepressor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptors (SMRT), by binding to negative HREs or by the direct repression of signal transcription factors on the active gene expression (Aranda & Pascual, 2001, Saijo *et al.*,

2013). These capabilities offer the NRs a particular role in the modulation of gene expression (Aranda & Pascual, 2001, Saijo *et al.*, 2013).

Furthermore, NRs modulate the gene programs not only by direct binding to HREs but also through signal transduction cross talk like the interference with AP-1 or NF-κB activities (Germain *et al.*, 2006). Transcription activity of NRs on modulated promotors will be regulated by a cyclic turnover of NRs (Germain *et al.*, 2006). This cycle seems to correlate with proteasome-dependent degradation activity and chromatin remodeling activity (Germain *et al.*, 2006).

1.3.5 NRs are modulators of the immune response in fungal infections

NRs are well-known actors in numerous biological roles like cell growth, differentiation and metabolic homeostasis (Aranda & Pascual, 2001, Jin & Li, 2010). In recent years, the focus of the effect of NRs in the immune response has increased (Baschant & Tuckermann, 2010, Hollm-Delgado *et al.*, 2014a, Hollm-Delgado *et al.*, 2014b, Khoo *et al.*, 2011b, Khoo *et al.*, 2012, Mayo-Wilson *et al.*, 2011, Nagy *et al.*, 2012, Nalbandian & Kovats, 2005, Nixon *et al.*, 2013). In fungal infection, several NRs have been suggested to play a crucial role in the outcome of invasive fungal infections: the GR, the PPARs, the ERs and the VDR (Gales *et al.*, 2010, Khoo *et al.*, 2011a, Lim *et al.*, 2015, Lionakis & Kontoyiannis, 2003, Majer *et al.*, 2012, Relloso *et al.*, 2012).

1.3.5.1 The glucocorticoid receptor (GR) in fungal infection

Corticosteroids, especially the synthetic forms are one of the most commonly used drugs in inflammatory diseases, autoimmune infections and malignancies (Busillo & Cidlowski, 2013, Cheng *et al.*, 2014). These lipophilic steroids, with cortisol as their representative member are ligands of the GR (NR3C1), secreted and regulated by the hypothalamic-pituitary-adrenal gland axis (Busillo & Cidlowski, 2013).The GR is well known for its anti-inflammatory regulatory capabilities like the repression of pro-inflammatory gene expression (Busillo & Cidlowski, 2013, Kassel & Herrlich, 2007, Kleiman *et al.*, 2012b, Nixon *et al.*, 2013). A lack of these important regulators by adrenalectomy or other causes of irregular production in steroids are associated with a higher mortality and low survival rate within sepsis and septic shock (Kleiman *et al.*, 2012a). Furthermore, an augmented endogenous production of corticosteroids, called the Cushing

syndrome or external intake of high-dose cortisol-derivate accompanied with increased long-term glucocorticoid level induces an impairment of immune cell activity (Lionakis & Kontoyiannis, 2003, Scheffel *et al.*, 2010). These patients are associated with a high risk in life-threatening invasive fungal infections (Lionakis & Kontoyiannis, 2003, Scheffel *et al.*, 2010).

1.3.5.2 **The estrogen receptors (ERs) in fungal infection**

ERs are steroid/thyroid hormone receptors, containing two subtypes: ERα (NR3A1) and ERβ (NR3A2), which are widely expressed in different types of tissues in male, female, including immune tissues and immune cells (Ascenzi *et al.*, 2006, Nalbandian & Kovats, 2005, Shi *et al.*, 2013). The best-known effector of the ERs is 17β-estradiol (Acconcia & Kumar, 2006, Ascenzi *et al.*, 2006). In acute inflammation, estradiol reduces inflammatory mediators induced by LPS, restores immunological homeostasis and prevents inflammatory gene transcription by inhibiting NF-κB intracellular transport (Ascenzi *et al.*, 2006, Choudhry *et al.*, 2007, Nalbandian & Kovats, 2005, Relloso *et al.*, 2012). Nevertheless, with high level of 17β-estradiol, fungi like *C. albicans* are allowed to flourish and increase their growth, as observed in urinary epithelial tissue (Relloso *et al.*, 2012). In humans, high level of 17β-estradiol is observed mainly in woman taking anti-contraceptive medication, during early pregnancy or during hormone replacement therapy (Relloso *et al.*, 2012). In these women, Relloso *et al*. observed a limited effect of the host immune response leading to an increased susceptibility to systemic candidiasis (Relloso *et al.*, 2012).

1.3.5.3 **The peroxisome proliferator-activated receptors (PPARs) in fungal infection**

The PPARs are well-known modulators of the glucose, lipid and amino acid metabolism as well as of the immune response (Nagy *et al.*, 2012, Rigamonti *et al.*, 2008). Three known subtypes of this NR are identified so far: PPARα (NR1C1), PPARδ (NR1C2) and PPARγ (NR1C3) (Matsuda *et al.*, 2013, Schoonjans *et al.*, 1996). PPARα and PPARγ are efficient modulators of the immune response in humans, PPARγ especially in invasive fungal infections (Gales *et al.*, 2010, Majer *et al.*, 2012, Marx *et al.*, 2004, Matsuda *et al.*, 2013, Nagy *et al.*, 2012, Rigamonti *et al.*, 2008). PPARs are generally expressed in

adipose tissue, colon and immune cells like the macrophages, important cells for the inflammatory regulation (Coste *et al.*, 2008, Majer *et al.*, 2012, Rigamonti *et al.*, 2008). Macrophages have multiple defence mechanism to eliminate *Candida* effectively (Coste *et al.*, 2008). The activation of PPARγ by its ligands, such as the synthetic agonist pioglitazone, leads to an increase of macrophage activity. Furthermore, PPARγ induces the transcription of antifungal PRRs like the Dectin-1 receptor (Coste *et al.*, 2008, Gales *et al.*, 2010).

In another study, Majer *et al.* observed an induction of the pro-inflammatory IFN I cytokine response in mouse monocytes during the challenge with invasive *Candida* infection (Majer *et al.*, 2012). With the help of the induced IFN I response, monocytes were capable to diminish this fungal invader (Majer *et al.*, 2012). However, an overstimulation of these cytokines led to a life-threatening stage with poor outcome (Majer *et al.*, 2012). The treatment with pioglitazone led into increased survival and restoration of immunological homeostasis (Majer *et al.*, 2012).

1.3.5.4 **The vitamin D receptor (VDR) in fungal infection**

Vitamin D deficiency is repeatedly reported to occur in critical ill patients, adults as well as children (Amrein *et al.*, 2014, McNally *et al.*, 2012). It is associated with a longer hospital stay and critical outcome (Amrein *et al.*, 2014, McNally *et al.*, 2012). The VDR (NR3I1) acts by its biological active ligand vitamin D_3 ($1.25(OH)_2D_3$) as well as by several synthetic agonists (Khoo *et al.*, 2011a, Mora *et al.*, 2008). The VDR is present in most immune cells (Khoo *et al.*, 2011a) and modulates a broad range of the immune responses of the innate and adaptive immune system, particularly in bacterial and fungal infection (Khoo *et al.*, 2011a, Khoo *et al.*, 2011b, Mora *et al.*, 2008, Nagy *et al.*, 2012, Youssef *et al.*, 2011). Khoo *et al*. observed a downregulation of the *C. albicans*-induced cytokine secretion in PBMCs in the presence of $1.25(OH)_2D_3$ in vitro (Khoo *et al.*, 2011a). This downregulation was mainly mediated by the inhibition of the expression of PRRs like Dectin-1, TLR2 and TLR4 at transcriptional and post-translational level (Khoo *et al.*, 2011a, Khoo *et al.*, 2011b, Nagy *et al.*, 2012). Furthermore, a recently released study by Lim *et al*. showed a dose- and target cell-dependent potential of vitamin D_3 to act both as an anti- and as a pro-inflammatory agent during fungal infections (Lim *et al.*, 2015).

Of 48 members of the NRs super family, only four NRs, the GR, ERs, PPARs and the VDR, are reported to play a role in fungal infections. Although many NRs are well known for their important status in the immune response, like the vitamin A receptor, nothing is known about its function in fungal infection.

1.4 VITAMIN A AND THE INTERACTION WITH ITS NRs: THE RETINOIC ACID RECEPTORS

1.4.1 General importance of vitamin A

Vitamin A and its derivatives, the retinoids, are important lipophilic mediators in a wide field of biological processes including cell development, cell differentiation, apoptosis and homeostasis (Alvarez *et al.*, 2014, Mora *et al.*, 2008). Furthermore, they are important mediators in vision, reproduction, embryonic growth, bone metabolism, haematopoiesis, maintenance of the skin and the cellular health, the brain function as well as modulators of the immune response (Alvarez *et al.*, 2014, Hall *et al.*, 2011, Mora *et al.*, 2008, O'Byrne & Blaner, 2013). Already in the 1920s, vitamin A was reported to be an anti-infective vitamin (Green & Mellanby, 1928, Semba, 1999). In 1925, Wolbach & Howe observed an atrophy of the spleen and the thymus gland in vitamin A deficient (VAD) rats (Wolbach & Howe, 1925). Furthermore, from the late ´90$_s$ until now, VAD children, especially in developing countries, were reported to show a higher risk of death from common childhood illness (Glasziou & Mackerras, 1993, Hollm-Delgado *et al.*, 2014b, Mayo-Wilson *et al.*, 2011, Pokhrel *et al.*, 1994, Sommer *et al.*, 1986). Moreover, in recent years, the immunological function of vitamin A has been in focus of intense clinical research (Hall *et al.*, 2011, Ho *et al.*, 2005, Ouziel *et al.*, 2013).

1.4.2 Uptake, metabolism and storage of vitamin A

Vitamin A has to be obtained through the diet, but can be stored within the body in relatively high doses (O'Byrne & Blaner, 2013). In humans, vitamin A is found either as the pro-vitamin A (the carotenoids like β-carotene), or as one of the preformed vitamin A metabolites (retinal, retinol, retinyl ester), and in low concentration as all-trans retinoic acid (atRA), the most active metabolite of

vitamin A (Alizadeh *et al.*, 2014, Alvarez *et al.*, 2014, O'Byrne & Blaner, 2013, Theodosiou *et al.*, 2010).

Preformed vitamin A is found in animal products and grains whereas highly pigmented vegetable products such as carrots, squash and yams mainly ensure the uptake of carotenoids (Alizadeh *et al.*, 2014, Theodosiou *et al.*, 2010). However, the bioavailability of retinol and carotenoids differs in humans (Burchum & Rosenthal, 2014). To measure the vitamin A activity, the retinol activity equivalents (RAE) is used as the most recent international standard, where vitamin A = µg retinol + (µg β-carotene equivalents/12)(Burchum & Rosenthal, 2014). 1 RAE equals 1µg retinol or 12 µg β-carotene (Burchum & Rosenthal, 2014). Nevertheless, various dietary factors like the food matrix or the specificity of transporters in the intestines have an effect on the bioavailability and bioaccessibility of carotenoids (Reboul *et al.*, 2006, van Het Hof *et al.*, 2000). In westernized countries, preformed vitamin A metabolites like retinol represent the main source of the vitamin A intake, in developing countries the main vitamin A source is covered by the uptake of carotenoids (Penniston & Tanumihardjo, 2006). It might be another cause of high incidence in VAD in developing countries.

For optimal intestinal absorption of the fat-soluble vitamin A, co-ingestion of dietary fat is required to enhance the uptake of preformed vitamin A metabolites and carotenoids (Harrison, 2012, Reboul, 2013). In the intestine at the mucosal epithelial barrier of the enterocytes, retinyl ester have to be hydrolysed at the luminal border to yield free retinol (O'Byrne & Blaner, 2013). Free and unesterified retinol can be directly absorbed by the enterocytes, enabled by the presence of efficient specific transporters (O'Byrne & Blaner, 2013, Reboul, 2013). Carotenoids are transported either unmodified via non-specific transporters or converted within the enterocytes into retinal by the BCMO1 enzyme (O'Byrne & Blaner, 2013, Reboul, 2013). This might explain the difference in the efficiency of retinol absorption (range between 75 % to 100 %) as compared to the less efficient absorption of carotenoids (from 3 % to 90 %) (Haskell, 2012, Reboul, 2013).

After the uptake within the enterocytes, all substrates will be transported to the hepatocytes by binding to chylomicrons (O'Byrne & Blaner, 2013).

From the liver, retinoids are distributed according to their need inside the body

(O'Byrne & Blaner, 2013). Several binding proteins stabilize and solubilize the lipophilic vitamin A metabolites: retinol binding proteins (RBPs), cellular retinol binding protein 1 (CRBP1), cellular retinoic acid binding proteins (CRABP1 and CRABP2) and albumin (Alizadeh *et al.*, 2014, Hall *et al.*, 2011). Retinol is the main transport form of the retinoids in the plasma, binding to the RBPs, circulating within the bloodstream of the body to provide the target cells with retinoids (Sun & Kawaguchi, 2011). Several controlling factors are existent to provide a constant blood concentration level of retinol including the enzymes that esterify retinol and hydrolyse retinol esters in the tissues (Ball, 2004, Penniston & Tanumihardjo, 2006). Retinol serves as a precursor of several active forms of the retinoids. It can be oxidized either reversibly in all-trans retinal or in all-trans retinoic acid (atRA) in an irreversible manner. Furthermore, retinol can be esterified to retinyl ester for further vitamin A storage in the stellate cells inside the liver (Nagy *et al.*, 2012, O'Byrne & Blaner, 2013, Ross *et al.*, 2011). Moreover, immunological tissues like spleen, thymus and lymph nodes consist of small amounts of retinyl ester, suggesting an important role of retinoids in immunological functions (Ross *et al.*, 2011). Retinyl esters have no known biological activity aside from retinol storage and serving as the substrate for the formation of the visual chromophore 11-cis-retinal (O'Byrne & Blaner, 2013).

All-trans retinal serves as an intermediate metabolite in the synthesis of all-trans retinoic acid (Nagy *et al.*, 2012). It derives either from retinol or β-carotenes by alcohol dehydrogenases, short chain reductases or β-carotene-15,15 ′-monooxygenase (BCMO1) (O'Byrne & Blaner, 2013).

For its biological activity, vitamin A has six known active metabolites, with atRA being the predominant physiological form for transcriptional regulation (Al Tanoury *et al.*, 2013, O'Byrne & Blaner, 2013, Theodosiou *et al.*, 2010). AtRA can either be produced inside the target cells, transferred to the nucleus by binding to CRABP I or CRABP II, or circulating in the plasma by binding to albumin (O'Byrne & Blaner, 2013). Nevertheless, because of its toxic character, retinol is the preferred transport form of retinoids in the blood circulation (Sun & Kawaguchi, 2011). Multiple factors seem to influence the generation of retinoic acid. So for example, atRA itself, fatty acids, TLR ligands and GM–CSF promote retinoic acid synthesis, whereas prostaglandin E2 inhibits its production (Alizadeh *et al.*, 2014, Kedishvili, 2013). Some immune cells have been reported

to generate atRA from plasma-retinol for paracrine action. This paracrine activity is important in the modulation of the innate and adaptive immune system, inducing mainly an anti- but also a pro-inflammatory response, depending on the concentration level of atRA (Abraham & Medzhitov, 2011, Hall *et al.*, 2011, Nagy *et al.*, 2012). Since atRA is known for its toxic character and high doses of retinoic acid are associated with teratogenicity and other side effects, the concentration of atRA within the cells is usually low and tightly regulated (Kedishvili, 2013, O'Byrne & Blaner, 2013). Furthermore, atRA cannot be recycled to retinol or retinal. It has to be degraded by several members of the cytochrome P450 enzyme family (Kedishvili, 2013, O'Byrne & Blaner, 2013).

1.4.3 Biological function of vitamin A and its NRs: the RARs

AtRA is the main mediator of vitamin A to modulate gene expression, either activating or repressing it (Al Tanoury *et al.*, 2013, O'Byrne & Blaner, 2013). Inside the target cells, atRA binds to the CRABP II, which mobilizes atRA to the nucleus. In the nucleus, atRA exerts its function by serving as an activating ligand of the retinoic acid receptors (RARs), which can form homodimers, but mainly forms heterodimers with retinoic x receptors (RXRs) (Altucci & Gronemeyer, 2001, Kedishvili, 2013, Osz *et al.*, 2012). For complete activation, the activated RAR: RXR dimer functions as a transcription factor, binds to the retinoic acid response elements (RAREs) in the promotor region of target genes and modulates transcription of these genes (Altucci & Gronemeyer, 2001, Cunningham & Duester, 2015, Ross, 2011). Nevertheless, the binding capability of the RARs are reported to differ depending on the dimeric partner. The homodimer RAR: RAR is reported to bind only high affine to the target genes if coactivators are present (Osz *et al.*, 2012, Walfish *et al.*, 1997). Nevertheless, the common RAR: RXR dimer, binds stably and with high affinity to the target genes in a non-permissive manner (Perez *et al.*, 2012, Ross *et al.*, 2011).

In an unliganded state, the RAR: RXR dimer associates with corepressor complexes such as NCoR1 or SMRT, which reside in or recruit complexes with HDACs activity while binding to the RAREs (Rochette-Egly & Germain, 2009). This association enables the complex to function in a repressive manner, for example by generating a condensed chromatin structure over the target promotor (Al Tanoury *et al.*, 2013, Rochette-Egly & Germain, 2009, Ross *et al.*, 2011).

Also other corepressors like Transducin-β-like 1 (TBL1) or other kinds of repressors such as Topoisomerase IIβ have been reported to interact with RAR target genes (Rochette-Egly & Germain, 2009). Nevertheless, the presence of atRA induces allosteric conformational changes in the LBD of the RAR dimer, leading to the dissociation of the corepressor complex (Al Tanoury *et al.*, 2013, Rochette-Egly & Germain, 2009). This step provokes an intensive binding of the RAR dimer to the target region, uniting further coactivators and large complexes with enzymatic activities, such as the decompactation of the chromatin (Al Tanoury *et al.*, 2013, Ross *et al.*, 2011). The classical coactivators bind to the surface of the RARs by a highly conserved LxxLL motif, whereas other co-regulators bind through other surfaces (Al Tanoury *et al.*, 2013). Once the chromatin is relaxed, the multiprotein complex consisting of the mediator, the DNA-dependent RNA – polymerase II, general transcription factors and nuclear excision repair factors (NER) is recruited to the promotor region, resulting in the initiation of the transcription (Al Tanoury *et al.*, 2013, Ross, 2011).

The termination of the transcription is realised either by degradation of RARs through the ubiquitin-protease complex and/or by recruitment of non-conventional coactivators with LxxLL motifs, such as RIP140 and PRAME which are associated to large complexes with chromatin repressing activity (Al Tanoury *et al.*, 2013).

Beside this mentioned direct, classical pathway, atRA uses other mechanisms to modulate the gene expression.

It uses direct mechanisms such as direct protein-protein interactions with other non-related transcriptions factors to activate or suppress the gene transcription (Harant *et al.*, 1996, Ho *et al.*, 2005). For example, retinoic acid acts as a negative regulator of the AP-1 responsive genes by the interaction of RARs with c-Jun, a member of the AP-1 transcription factor complex resulting in prevention of AP-1 binding to its response element (Harant *et al.*, 1996, Ho *et al.*, 2005). Furthermore, several indirect regulatory mechanism of retinoic acid in gene expression exists. For example, the induction of an intermediary (usually a transcription factor), which is directly responsible for the effect, like the NFκB-complex in immunological processes, the modulation of mRNA stability and the interaction with NRs other than the classical RAR: RXR dimers (Bianchi *et al.*, 2009, Dai *et al.*, 2004, Harant *et al.*, 1996). Beside these mentioned modulatory

effect which atRA exerts inside the nucleus, additional extra nuclear, non-transcriptional effects of retinoic acid are existent. AtRA is capable to interfere rapidly and transiently through non-genomic effects (Al Tanoury *et al.*, 2013). These non-genomic effects include the modulation of the MAPK or Janus kinase-signalling pathway by the use of phosphorylation processes or the modulation of other NRs like the PPARs (Al Tanoury *et al.*, 2013). Interestingly, it has been shown that these retinoic acid-induced kinases cross talk with the transcriptional activity of RARs and other transcription factors (Al Tanoury *et al.*, 2013). This interference mainly takes place through phosphorylation processes that influences their DNA recruitment, timings of the sequential recruitment of different classes of co-regulators as well as the stability of the phosphorylated proteins (Al Tanoury *et al.*, 2013).

Even RARs and RXRs are phosphoproteins (Al Tanoury *et al.*, 2013). For instance, RARγ can interact in a non-phosphorylated state with vinexin-β, an adaptor protein which represses the RARγ-mediated transcription by inhibiting the binding of RARγ to the DNA and its sequestration out of chromatin (Al Tanoury *et al.*, 2013). This wide range of potential mechanisms of vitamin A to modulate genes suggesting a wide spreading nature of vitamin A in biological functions (Al Tanoury *et al.*, 2013).

1.4.4 Clinical importance of vitamin A in the immune system

The immunological function of vitamin A has been in focus of intense clinical research in the recent years (Hall *et al.*, 2011, Ho *et al.*, 2005, Ouziel *et al.*, 2013). Vitamin A has been shown to reduce excessive inflammatory monocyte response observed in patients with acne (Dispenza *et al.*, 2012, Ouziel *et al.*, 2013). Retinoids and their synthetic isoforms are already widely used against pro-inflammatory skin diseases and different malignancies (Alizadeh *et al.*, 2014, Altucci & Gronemeyer, 2001, Dispenza *et al.*, 2012). Furthermore, plasmatic vitamin A deficiency has been correlated with scores of severity and increased inflammatory immune response in alcoholic liver disease (ALD) patients. Ouziel *et al.* observed a decrease in the overproduction of TNF-α in LPS-stimulated PBMCs of ALD patients after pre-treating these immune cells with atRA in *vitro* (Ouziel *et al.*, 2013). Furthermore, Ouziel *et al.* investigated the impact of vitamin A in *vivo* in VAD mice (Ouziel *et al.*, 2013). He observed a decrease in

the activation of peritoneal macrophages after supplementing these mice with vitamin A (Ouziel *et al.*, 2013). In infections of bacterial, protozoan and viral origin, treatment with vitamin A showed a protective effect (Hollm-Delgado *et al.*, 2014b, Mayo-Wilson *et al.*, 2011, Semba, 1999). Retinoic acid suppresses the production of several pro-inflammatory cytokines such as IL12 and mediators like the NO in LPS-activated macrophages (Pino-Lagos *et al.*, 2008). Furthermore, Austenaa *et al.* showed an inhibitory effect of retinoic acid on the LPS-induced NF-κB activity in the human myeloblastic cell line U937 (Austenaa *et al.*, 2009). Moreover, vitamin A plays an immunomodulatory role not only in the innate immune system but also in the adaptive immune system (Mora *et al.*, 2008). It has been shown to induce the overproduction of the pro-inflammatory cytokine IL6 by activated B-cells, to exert a B-cell isotype switch and antibody production either in a direct or indirect effect (Pino-Lagos *et al.*, 2008). In T-cells, retinoic acid induces in the presence of TGF-β the conversion from naive T-cells into regulatory T-cells, which are important for immune system homeostasis and prevention of autoimmune diseases (Liu *et al.*, 2015, Raverdeau & Mills, 2014). Furthermore, RA regulates the differentiation of T_H cells (Iwata *et al.*, 2003, Liu *et al.*, 2015, Raverdeau & Mills, 2014). It is reported to promote the anti-inflammatory T_{H2}-cell response, while inhibiting the pro-inflammatory T_{H1}-cell response as well as the T_{H17} cell induction in an inflammatory setting (Iwata *et al.*, 2003, Liu *et al.*, 2015, Raverdeau & Mills, 2014).

In intra-abdominal sepsis, Demetriou *et al*. showed a significant protective effect of vitamin A in adult male rats (Demetriou *et al.*, 1984).

Since secondary infections due to post-sepsis immunosuppression are a major cause of death in patients, Martire-Greco *et al.* investigated the effect of atRA on LPS-immunosuppressed mice (Martire-Greco *et al.*, 2014). The treatment with atRA restored T-cell proliferation and showed an increase in $CD4^+$- and $CD8^+$ T_H-cells.

Summarizing, vitamin A seems to have an impact on several parts of the immune system (Al Tanoury *et al.*, 2013, Bianchi *et al.*, 2009, Harris *et al.*, 2004, Liu *et al.*, 2015, Pino-Lagos *et al.*, 2008, Raverdeau & Mills, 2014, Theodosiou *et al.*, 2010).

Nevertheless, in fungal infection, the effect of vitamin A is still unclear.

Since fungal sepsis is still on a rise, in the present work we wanted to investigate the impact of vitamin A in the immune response in *C. albicans* infections.

2. HYPOTHESIS

1. Vitamin A may modulate the pro-inflammatory immune response in monocytes upon *C. albicans* infection.
2. Vitamin A may modulate the expression levels of fungal-relevant PRRs such as Dectin-1 and its co-receptors, as well as the Dectin-1-dependent immune response in monocytes.
3. The immunomodulatory effect of vitamin A during *C. albicans* infections might be RAR-dependent.

2.1 OBJECTIVES

1. To investigate the effect of vitamin A on the expression pattern of the cytokines TNFα, IL6, IL12b and IL10 in monocytes upon stimulation with *C. albicans* or β-1.3 glucan beads. In parallel, we aim to investigate the potential modulatory role of atRA on the IFN I pathway.
2. To analyse the impact of vitamin A on the expression of fungal-relevant PRRs such as Dectin-1 and its co-receptors TLR2 and Gal-3.
3. To elucidate whether the effect of vitamin A is in each case RAR-dependent by making use of specific agonists and antagonist of the different potential receptors.

3. MATERIALS AND METHODS

3.1 MATERIALS: find attached materials used during the thesis (Tables 1-10).

Every buffer and solution was prepared with distilled or double distilled water. The pH-value was adjusted by either hydrochloride acid or sodium hydroxide.

Tab. 1: Materials used for cultivation of *C. albicans*

Components	Ingredients	Manufacturer
YPD-medium	Glucose 20g/ l	Carl Roth, Germany
	Peptone 20g/ l	Carl Roth, Germany
	Yeast extract 10g/ l	Merck, Germany
	Millipore water up to 1 l	Merck, Germany
	pH 5	
YPD- agar	Contents as YPD medium plus	
	Bacteriological agar 20g/ l	Sigma Aldrich, Germany

Tab. 2: Assessment of cell viability and cell count

Components	Manufacturer
Trypan Blue 0.4 %	Invitrogen, Germany
Neubauer-counting chamber	VWR International GmbH, Germany
Propidium Iodide, 0.1 mg/ ml	Life Technologies, Germany

Tab. 3: Materials used for primary cell isolation

Components	Manufacturer
Leukosep Falcon 50 ml	Greiner Bio-One GmbH, Germany
MACS- Monocyte Isolation Kit II	Miltenyi Biotec, Germany
MidiMACS Separator	Miltenyi Biotec, Germany
LS Columns	Miltenyi Biotec, Germany
DBPS pH 7	Invitrogen, Germany
RPMI 1640 GlutaMAX ™	Invitrogen, Germany

Ficoll-Paque Plus	GE-Healthcare, Germany
ACK Erythrocyte Lysing Buffer	Invitrogen, Germany
NaCl 0.45 % , autoclaved	Carl Roth, Germany
EDTA, 0.1 M, sterile filtered	Sigma Aldrich, Germany
Fetal calf serum (FCS), heat inactivated	Biochrom GmbH, Germany
Penicillin-Streptomycin	Invitrogen, Germany

Tab. 4: Materials used for cell-culture in stimulation assay

Components	Manufacturer
Microplate 6-well	VWR International Gmb, Germany
RPMI 1640 GlutaMAX ™	Invitrogen, Germany
DMSO	Sigma Aldrich, Germany
Penicillin-Streptomycin	Invitrogen, Germany
Ethanol 99.8 %	Sigma Aldrich, Germany

Tab. 5: Materials for RNA-isolation and reverse transcription

5.1 Materials for RNA-isolation

Components	Manufacturer
Ethanol, 99.8 %	Nordbrand Nordhausen GmbH, Germany
Nuclease free water	Invitrogen, Germany
RNeasy Mini Kit	Qiagen GmbH, Germany
RNase-free DNase Set	Qiagen GmbH, Germany
Cell-scraper	VWR International GmbH, Germany
DNA LowBind Tubes	Eppendorf AG, Germany
Sterilfilter 3.8 µm/ cm^2	Carl Roth, Germany
Filtertips, ep Dualfilter T.I.P.S.	Eppendorf AG, Germany

5.2 Material for reverse transcription from RNA into cDNA

Components	Manufacturer
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, UK
Nuclease free water	Invitrogen, Germany
Tris-EDTA (TE) pH 7.4; 0.5 x	FLUKA, Germany
S1000™ Thermal cycler	BioRad, UK
CAS Robotics 1200	Qiagen, Germany

DNA LowBind Tubes	Eppendorf AG, Germany
Filtertips, wide bore	Quiagen, Germany
CAS Robotics 1200	Qiagen, Germany

Tab. 6: Materials for semi-quantitative PCR and gel electrophoresis

Components	**Manufacturer**
PCR dNTP Mix	Promega GmbH, Germany
Agarose, LE analytical grade	Promega GmbH, Germany
Ethidium bromide (10 mg/ ml)	Sigma Aldrich, Germany
Quick Load 100bp DNA Ladder 50 µg/ ml	New England Biolabs GmbH, Germany
S1000^{TM} Thermal cycler	BioRad, UK

Components	**Ingredients**
1x TBE-Buffer	89 mM TRIS
	89 mM Boric acid
	1 mM EDTA
	pH 8
1.7 % Agarose gel	1.7 % Agarose LE analytical grade
	2.5 ug/ 100 ml ethidium bromide

Tab. 7: Materials for real-time quantitative PCR (RT-qPCR)

7.1 Components for RT-qPCR

Components	**Manufacturer**
Nuclease free water	Invitrogen, Germany
Tris-EDTA (TE) pH 7.4; 0.5 x	FLUKA, Germany
RNeasy Mini Kit	Qiagen GmbH, Germany
RNase-free DNase Set	Qiagen GmbH, Germany
Cell-scraper	VWR International GmbH, Germany
SensiMix SYBR No-ROX Kit	Bioline, Germany
DNA LowBind Tubes	Eppendorf AG, Germany
Sterilfilter 3.8 µm/ cm^2	Carl Roth, Germany

Filtertips, ep Dualfilter T.I.P.S.	Eppendorf AG, Germany
Filtertips, wide bore	Qiagen, Germany
CAS Robotics 1200	Qiagen, Germany

7.2 Primer – of genes used in this study

Humane Gene	***Symbol***
Peptidylpropyl Isomerase B	***PPIB***
Hypoxanthine Phosphoribosyltransferase 1	***HPRT1***
Glyceraldehyd-3 Phosphat Dehydrogenase	***GAPDH***
Tumor necrosis factor alpha	***TNFα***
Interleukin 6	***Il6***
Interleukin 12 subunit beta	***IL12b***
Interferon beta 1	***IFNβ1***
Dectin-1	***Dectin-1***
Galectin 3	***Gal-3***
Toll-like receptor 2	***TLR2***
Toll-like receptor 4	***TLR4***
Toll-like receptor 7	***TLR7***
Toll-like receptor 9	***TLR9***
Retinoid x receptor alpha	***RXRA***
Retinoid x receptor beta	***RXRB***
Retinoid x receptor gamma	***RXRG***
Retinoic acid receptor alpha	***RARA***
Retinoic acid receptor beta	***RARB***
Retinoic acid receptor gamma	***RARG***
Suppressor of cytokine signalling 1	***SOCS1***
Suppressor of cytokine signalling 3	***SOCS3***
CD14 molecule	***CD14***
Mitogen-activated protein kinase phosphatase 1	***MKP1***
Interferon receptor 1	***IFNAR***
interferon regulatory factor 1	***IRF1***
interferon regulatory factor 3	***IRF3***

interferon regulatory factor 5	***IRF5***
interferon regulatory factor 7	***IRF7***
interferon regulatory factor 9	***IRF9***
Signal transducer and activator of transcription 1	***STAT1***
Signal transducer and activator of transcription 2	***STAT2***

For sequence of forward and reverse primer pair of indicated target genes and expected size of the PCR product see Figure 4.

Tab. 8: Cell media

8.1 Medium for monocyte isolation

Components	**Manufacturer**
DBPS	Invitrogen, Germany
EDTA, sterile filtered, 2 mM	Invitrogen, Germany
FCS, 0.5 %, heat- inactivated	Invitrogen, Germany

8.2 Medium for cell culture

Primary cell stimulation assay

Components	**Manufacturer**
RPMI 1640 GlutaMAX ™	Invitrogen, Germany
Penicillin-Streptomycin, 1 %	Invitrogen, Germany

THP-1 cell culture medium

Components	**Manufacturer**
RPMI 1640 GlutaMAX ™	Invitrogen, Germany
Penicillin-Streptomycin, 1 %	Invitrogen, Germany
FCS, 10 %, heat- inactivated	Biochrom GmbH, Germany

THP-1 cell stimulation assay

Components	**Manufacturer**
RPMI 1640 GlutaMAX ™	Invitrogen, Germany
Penicillin-Streptomycin, 1 %	Invitrogen, Germany

8.3 Medium for flow cytometry-analysis

Buffer for flow cytometry

Components	Manufacturer
DBPS pH 7,	Invitrogen, Germany
- supplemented with 2 % FCS	Biochrom GmbH, Germany

Tab. 9: Antibodies and synthetic agonists/ antagonists for stimulation assay

9.1 Antibodies for flow cytometry

Human Dectin1/ CLEC7A	Ingredients	Manufacturer
Primary antibody	Monoclonal-mouse-anti-human Anti-Dectin1 Clone 259931, (MAB1859) Working concentration: 1 µg/ 2 µl	R&D Systems, Germany
Secondary antibody	Goat-anti-mouse IgG, APC labelled Working concentration: 1 µg/ 2 µl	BD, Germany
Isotype Control	Mouse Isotype IgG2b K eBMG2b	eBioscience, Germany

Human TLR2	Ingredients	Manufacturer
Primary antibody	Monoclonal mouse-anti-human Anti-TLR2, FITC conjugated Clone T2.5, (ab59711) Working concentration: 0.2 µg/ 2 µl	Abcam, US
Isotype Control	Mouse IgG1 K Isotype Control FITC conjugated Clone: P3.6.2.8.1 Working concentration: 1 µg/ 2 µl	eBioscience, Germany

9.2. Synthetic agonists/ antagonists

9.2.1 TLR-agonists for the PBMC co-fungal stimulation assay

TLR- agonists	Ingredients	Manufacturer
TLR7-agonist	Imiquimod - Imidazoquinoline	Biomol GmbH, Germany

	Amine analogue to guanosine Synthetic human TLR7-agonist Working concentration: 5 µg/ ml Diluted in DMSO	
TLR9-agonist	CpG Type A–Oligonucleotide Type A (ODN2336) Synthetic human TLR9-agonist Working concentration: 10 µg/ ml Diluted in water	InvivoGene Biotech, China

9.2.2 Retinoic acid receptor (RAR) stimulation assay

Retinoic acid receptor-agonists		**Manufacturer**
RARα-agonist	BMS753 Working concentration: 1 µM	Tocris Bioscience, UK
RARγ-agonist	BMS961 Working concentration: 1 µM	Tocris Bioscience, UK

Retinoic acid receptor-antagonists		**Manufacturer**
RARα-antagonist	BMS195614 Working concentration: 1 µM	Tocris Bioscience, UK
RARγ-antagonist	MM11253 Working concentration: 1µM	Tocris Bioscience, UK

Tab. 10: Materials for ELISA

Chemokine	**ELISA-Kit**	**Manufacturer**
TNFα	human TNFα ELISA-Kit	Thermo Scientific Pierce Biotechnology, US
IL6	human IL6 ELISA-Kit	eBioscience, Germany
IL12b	human ELISA IL12b	Hölzel Diagnostika GmbH, Germany
IL10	human IL10 ELISA-Kit	eBioscience, Germany
IFNβ	human IFN-β ELISA-Kit	Thermo Scientific Pierce Biotechnology, US

Humane Gene	*Symbol*	Forward Primer	Reverse Primer	Size (bp)
Peptidylpropyl isomerase B	***PPIB***	ATGTAGGCCGGGTGATCTTT	TGAAGTTCTCATCGGGGAAG	219
Hypoxanthine Phosphoribosyltransferase 1	***HPRT1***	GACCAGTCAACAGGGGACAT	AACACTTCGTGGGGTCCTTTTC	195
Glyceraldehyd-3 phosphat Dehydrogenase	***GAPDH***	ATGACCCCTTCATTGACCTC	GCATCGCCCCACTTGATTTT	169
Tumor necrosis factor alpha	***TNFα***	TTCTCCTTCCTGATCGTGGC	ACTCGGGGTTCGAGAAGATG	150
Interleukin 6	***Il6***	GAGGAGACTTGCCTGGTGAA	TGGGTGAGGGGTGGTTATTG	186
Interleukin 12, subunit beta	***IL12b***	ACAACATCGGTTTCAGGGCCA	GGTCCAAGGTCCAGGTGATA	266
Interferon beta 1	***IFN-β***	TGCTCTCCTGTTGTGCTTCT	CCACAGGAGCTTCTGACACT	103
Dectin-1	***Dectin-1***	ACACTTCGACTCTCAAAGCA	TACAGCAATGAGGCGCCAA	91
Galectin 3	***Gal-3***	CCCATCTTCTGGACAGCCAA	CTTCACCGTGCCCAGAATTG	151
Toll-like receptor 2	***TLR2***	TGCATTCCCAAGACACTGGA	AGGGAGGCATCTGGTAGAGT	131
Toll-like receptor 4	***TLR4***	CAACCTCCCCTTCTCAACCA	CTGGATGGGGTTTCCTGTCA	196
Toll-like receptor 7	***TLR7***	ATCTTGGCACCTCTCATGCT	ACCATCTAGCCCCAAGGAGT	158
Toll-like receptor 9	***TLR9***	GAAGGGACCTCGAGTGTGAA	CTCACAGGGTAGGAAGGCAG	196
Retinoid x receptor alpha	***RXRA***	GCGCCATCGTCCTCTTTAAC	TGCTCTGGGTACTTGTGCTT	121
Retinoid x receptor beta	***RXRB***	TACTCTTGCCGGGACAACAA	GCCCTGGTCACTCTTCTGTT	240
Retinoid x receptor gamma	***RXRG***	TGCAGATGGACAAGTCGGAA	TTCAAGCCAATGGAACGCAG	214
Retinoic acid receptor alpha	***RARA***	CCACATGTTCCCCAAGATGC	GCCCTGTGAGTTCTCCAACA	145
Retinoic acid receptor beta	***RARB***	TCGTCTGCCAGGACAAATCA	TGGGCATCGATTCCTGGTGA	158
Retinoic acid receptor gamma	***RARG***	CAAGGTCAGCAAAGCCCATC	ACTTGGTAGCCAGCTCAGTG	137
Suppressor of cytokine Signalling 1	***SOCS1***	TCCCCTTCCAGATTTGACCG	GGAGGGTACCCACATGGTT	103
Suppressor of cytokine Signalling 3	***SOCS3***	GAGACTTCGATTCGGGACCA	GGAAACTTGCTGTGGGTGAC	124
CD14 molecule	***CD14***	GCAGCCGAAGAGTTCACAAG	ATCGTCCAGCTCACAAGGTT	206
Mitogen-activated protein Kinase phosphatase 1	***MKP1***	CCTTTCTGTACCTGGGCAGT	GCAGTGGACAAACACCCTTC	239
Interferon receptor 1	***IFNAR***	CTCCGCGTACAAGCATCTGA	AGGCGTGTTTCCAGACTGTT	168
Interferon regulatory factor 1	***IRF1***	AACATGCCCATCACTCGGAT	TGGGATCTGGCTCCTTTTCC	226
Interferon regulatory factor 3	***IRF3***	CTGCCAACCTGGAAGAGGAA	TGGGAAAAGTCCCCAACTCC	140
Interferon regulatory factor 5	***IRF5***	AGTACCCAGGGCTTCAATGG	AGTCCCGGCTCTTGTTAAGG	213
Interferon regulatory factor 7	***IRF7***	ACTGTGACACCCCCATCTTC	TCGTCATAGAGGCTGTTGGC	277
Interferon regulatory factor 9	***IRF9***	ATAAGGAGGGGGACACAGGA	ACTTTCTGAGTCCCTGGCTG	181
Signal transducer and activator Of transcription 1	***STAT1***	ACGCTGCCAATGATGTTTCA	ATCTCTGGGCGTTTTCCAGA	231
Signal transducer and activator Of transcription 2	***STAT2***	TCACAGAGTTGCTACAGCGT	TTGCCTTCCTGGAGTCTCAC	151

Fig. 4: Sequence of forward and reverse primer pair of indicated target genes and expected size of the polymerase chain reaction (PCR) product

3.2 METHODS

3.2.1 *C. albicans*

For our study, the virulent wild-type strain SC5314 of *C. albicans* was used, kindly provided by Fungal Septomics Jena, Germany, stored at -80 °C (Gillum *et al.*, 1984).

Culture media: *C. albicans* was cultivated in YPD-agar plates and YPD-medium for optimal growth of the fungal yeasts. Both media were prepared as described in table 1 and autoclaved before use. YPD-agar plates were stored at 4 °C, the liquid YPD-medium at room temperature.

Re-cultivation of *C. albicans* yeasts: For re-cultivation of *C. albicans*, the frozen fungus was stored in dry ice to protect from thawing. In sterile conditions, the fungus was spread over a YPD-agar plate by using a sterile inoculating loop to obtain single isolated colonies. The inoculated agar was incubated for 24 hours at a controlled temperature of 30 °C and 0.04 % CO_2 according to previous studies (Carlisle *et al.*, 2009, Mayer *et al.*, 2012, Wang, 2009).

C. albicans isolation: Overnight fungal yeast culture were grown in YPD-medium at aerobe conditions at 0.05 % CO_2, 30 °C and 210 rpm in the incubator shaker series 126 (New Brunswick Scientific) to multiply itself in numbers (Mayer *et al.*, 2012).

Immediately before stimulation of monocytes with *C. albicans,* the *C. albicans* yeast culture was washed three times and re-suspended in DPBS at a final concentration of 10^8 yeast/ ml. To avoid overbalanced growth of *C. albicans* regarding to our preliminary results and decrease of monocyte survival due to hyphal formation of living *C. albicans* we next inactivated the fungal yeasts. The UV-inactivation was performed in a UVC 500 – Crosslinker (Amersham,UK) using two doses of 100 mJ/ cm^2 immediately before stimulation (Esteban *et al.*, 2011). Due to preliminary alteration in dosage in a survival curve of *C. albicans*, the dosage of UV-inactivation could be minimized to two times of 100 mJ/ cm^2 at a concentration of 1 * 10^8 yeasts/ ml DPBS. The morphological shape did not alter.

Counting and observation of viability was performed by tryphan blue staining and microscopic observation (Primovert by Zeiss, Germany).

3.2.2 Monocyte and PBMC isolation

Human monocytes were isolated from buffy coats of healthy male volunteers kindly provided by Dr. Dagmar Barz of the Institute of Transfusion Medicine at Jena University

Hospital, Germany. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation according to manufacturer's instructions (Ferrante & Thong, 1980). Primarily, buffy coat diluted 1:4 with DBPS (Invitrogen, Germany) was layered on an equal volume of Ficoll hypaque Plus solution (GE Healthcare , Germany) and centrifuged in 50 ml sterile Leukosep tubes with porous barrier (Greiner-Bio-One GmbH, Germany) at 800 g for 15 min without acceleration or brake (Ferrante & Thong, 1980). After centrifugation, a proper density gradient was observed as follows: a serum phase, a clear white PBMC band, and a red sediment, consisting mainly of erythrocytes, and granulocytes (Fig. 5).

Fig. 5: PBMC-isolation from buffy coat before and after density gradient centrifugation

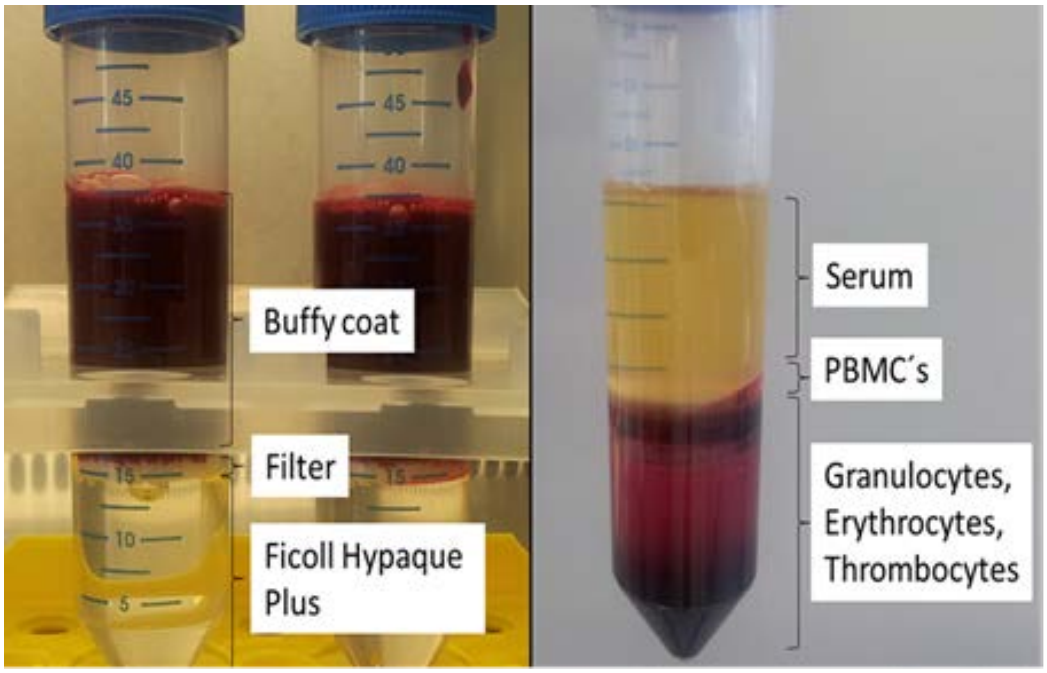

Fig. 5: The figure shows PBMC-isolation before (left) and after (right) density gradient centrifugation. PBMC-isolation was enabled by 1:4 DPBS dilution and further Fiqoll hypaque Plus supply.

The PBMC-layer was collected in 50 ml falcon tubes by sterile pasteur pipettes and washed with equal amount of sterile, 4 °C cold, 0.45 % NaCl. The next step was performed to clear away remaining erythrocytes by the use of a hypotonic buffer (ACK Erythrocyte Lysing Buffer, Invitrogen, Germany) according to manufacturer´s instruction. After a washing step with DPBS the reddish cell pellet turned white. These last two steps were repeated if necessary.

Two more washing steps with DPBS were included to sluice remaining thrombocytes. Then, PBMCs were used either directly for further experiments, adjusted to a

concentration of 5 * 10^6 cells/ ml, or used for monocyte isolation.

Untouched monocytes were isolated with the help of a MACS system, which couples negative labelling using biotin-conjugated antibodies with magnetic sorting using anti-biotin beads. Consequently, highly enriched $CD14^+$ unlabelled monocytes were collected. The purity of the obtained monocytes was higher than 92 % assessed by $CD14^+$ labelling and flow cytometry analysis (Fig. 6). Viability of PBMCs and isolated monocytes was > 95 %, as proven by microscopic observation in a Neubauer-haemocytometer including trypan blue staining.

Fig. 6: $CD14^+$ monocytes after magnetic separation from PBMCs verified by flow cytometry

Fig. 6: Verification of the purity in monocyte samples after magnetic separation of PBMCs by CD14- stained cells (FITC labelled). Figure shows a flow cytometry plot of monocytes with high purity of $CD14^+$ cells (>95%) after magnetic separation of PBMCs.

3.2.2.1 **Monocyte stimulation assay**

In the monocyte stimulation assay, monocytes are challenged with UV-inactivated *C. albicans* yeast in the presence or absence of 1 µM atRA to investigate the impact of vitamin A at transcriptional and post-translational level of the *C. albicans*-induced immune response. After monocyte isolation, monocytes were dispersed at 4 * 10^6 cells/ ml RPMI GlutaMax™ medium (Invitrogen, UK) containing 1 % Penicillin/

Streptomycin (Invitrogen, UK) in 2 ml/ well in 6 well plates (VWR International, Germany). Cells were allowed to equilibrate at 37 °C, 5 % CO_2 for 1 hour in the incubator (Galaxy 170S, New Brunswick, Eppendorf Company). Then, cells were pre-stimulated for 30 minutes with either 1 µM atRA or specific RARα- or RARγ–agonists/ -antagonists, followed by the addition of freshly prepared inactivated *C. albicans* yeast at a monocyte–fungus ratio of 1:1. Additionally we used unstimulated monocytes for internal control as well as one control of inactivated *C. albicans* and one of live *C. albicans* yeast at the same ratio. To assess the impact of vitamin A on the specific Dectin-1 signalling pathway in monocytes, we used a Dectin-1 agonist monocyte ratio of 5: 1 involving β-1.3 glucan coated beads as specific ligands of the Dectin-1 receptor. In all experiments we used an internal unstained control with 3 µl of 99.8 % ethanol, sterile filtered. Monocytes were incubated for 5 hours and 16 hours at 37 °C and 5 % CO_2. Viability of the cells was above 90 % at both times junctures, assessed by trypan-blue staining. After 5 hours and 16 hours stimulation, culture supernatants were frozen at -80 °C for further ELISA-analysis. Monocytes were harvested for subsequent RNA-isolation. Similar stimulation procedure was used for all experiments involving flow cytometry measurements, although in this case only 1.5 million monocytes were used and the stimulation periods were 12 hours, 18 hours and 24 hours.

3.2.2.2 **PBMC stimulation assay**

After isolation, PBMCs were apportioned at a concentration of 5 * 10^6 cells/ ml in RPMI GlutaMax medium (Invitrogen, UK) supplemented with 1 % Penicillin/ Streptomycin (Invitrogen, UK) in an amount of 2 ml/ well in 6 well plates (VWR International, Germany) for RNA-Extraction. Cells were allowed to equilibrate for 1 hour in the incubator (Galaxy 170S, New Brunswick, Eppendorf Company) at 37 °C, 5 % CO_2. In the next step, cells were pre-stimulated for 1 hour either with a specific TLR7- or TLR9-agonist alone or in combination with 1 µM atRA. The TLR9-agonist CpG, Type A was used by an amount of 10 µg/ ml diluted in sterile filtered water. The TLR7-agonist Imiquimod was only available diluted in DMSO, used with a concentration of 5 µg/ ml. After 1 hours of stimulation just freshly prepared UV-inactivated *C. albicans* yeast at a monocyte–fungus ratio of 1:1 was added as well as one control of inactivated and one control of live *C. albicans* yeast at the same ratio.

In all experiments an internal unstained control with 3 µl of 99.8 % ethanol, sterile filtered or with 3 µl of DMSO for Imiquimod was included. PBMCs were incubated for

5 hours at 37 °C and 5 % CO_2. The viability of the cells was above 95 % proven by trypan-blue staining. After 5 hours of stimulation, PBMCs were harvested for further RNA-analyses.

3.2.3 Preparation of the all-trans retinoic acid

All-trans retinoic acid (atRA), the natural agonist of the retinoic acid receptors (RARs) was used at a concentration of 1 µM based on previous studies and preliminary results (Cho *et al.*, 2011, Li *et al.*, 2003, Liu *et al.*, 2000). The provided lyophilic form (Sigma Aldrich, Germany) of atRA was dissolved in 99.8 % ethanol and sterile filtered. Several aliquots of 10 mM atRA were stored at -20 °C under dark conditions for not more than 3 months. Due to its high sensitivity to light, heat and air, a new aliquot was taken for each experiment. Immediately, before the stimulation assay, a working concentration of 1 mM atRA was prepared.

3.2.4 Preparation of RAR- agonists/ -antagonists

The selective RARγ- agonist, -antagonist as well as the selective RARα-agonist and -antagonist (Table 9.2.2) were dissolved in 99.8 % ethanol, sterile filtered and used at a concentration of 1 µM.

3.2.5 Preparation of β-1.3 glucan beads

β-1.3 glucan beads kindly provided by Massachusetts General Hospital, USA were prepared as previously described (Tam *et al.*, 2012). Before the use of the received β-1.3 glucan beads in the monocyte stimulation model, we wanted to verify the purity of the β-1.3 glucan beads. Therefore, we incubated HEK 293 cells with the beads and analysed for any changes at transcriptional level similar to the induction by LPS.
No contamination could be found.

3.2.6 Preparation of TLR-agonists

All TLR-agonists (Table 9.2.1) were present in lyophilic form. Immediately before stimulation, the TLR9-agonist CpG Type A was dissolved in water, whereas Imiquimod was dissolved in DMSO. Solvent controls for ethanol and DMSO were included in all experiments.

3.2.7 **RT-PCR and semi-quantitative PCR**

3.2.7.1 **RNA-Isolation**

To analyse the gene expression of the target genes after 5 hours and 16 hours of stimulation, total RNA was isolated from 8 * 10^6 monocytes or 10 * 10^6 PBMCs / stimulated population by RNeasy mini kit (Qiagen, Germany). An additional step of RNA-isolation by the use of DNAse I was included to prevent impurity with genomic DNA. First of all, the culture medium was transferred by pipets into RNase free DNA low bind collection tubes and centrifuged at 1000 g for 5 min. Centrifuged supernatants were transferred into a new column leaving a cell pellet on the bottom. Supernatants were stored immediately at -20 °C for further post-translational analyses.

Meanwhile, 600 µl RLT-Lysis buffer was added into each well, scrapped carefully to obtain RNA of leftover monocytes. This RLT buffer cell-mixture was added to the equivalent cell pellet compatible to the stimulation, homogenized thoroughly.

For the next isolation steps, the RNA needs to bind to a silica membrane in RNeasy mini spin columns, followed by several washing steps to distinguish all contaminants. Therefore, an equal amount of 70 % of ethanol was added to the RLT buffer cell-mixture and homogenized thoroughly again, before adding to the spin column.

Next, a centrifugation step of 30 seconds was included. Then, collection columns were changed to distinguish remaining guanidine. Guanidine is capable to interfere and to inhibit the activity of the polymerase enzyme. Further purification of the RNA was realized by the use of several different wash buffers in accordance to manufacturer´s instruction. Between each washing step columns were centrifuged at 9000 g. Furthermore, a DNAse I incubation step was included to prevent contamination with genomic DNA, followed again by a centrifugation of 9000 g. In the last step of the RNA-isolation, 40 µl of purified mRNA was collected in DNA low bind columns, immediately stored at -20 °C for 2 days, and further stored at -80 °C. To protect the RNA, nuclease-free tubes were used in all experiments and reactions handling with RNA.

3.2.7.2 **Quantification of purified mRNA**

The NanoDrop D-1000 Spectrometer (Thermo-Fisher Scientific, Germany) was used to obtain the amount and quality of the isolated RNA, using 2 ul RNA/ sample (Nolan *et al.*, 2006). After examination, the RNA was stored at – 80 °C.

Nucleotides, RNA, ssDNA, and dsDNA, all absorb at a wavelength of 260 nm. Phenolic

compounds and other contaminants such as guanidine isothiocyanate absorb at ~230 nm, whereas peptides and proteins at ~280 nm, concluding that contaminated RNA is mainly seen in wavelengths of 260 nm as well as 230 nm. The ratio of 260/ 280 in pure RNA averages between 1.8-2.0, whereas the ratio of 260/ 230 indicates pure nucleic acid at ~2 or above (Chomczynski & Sacchi, 2006, Fleige & Pfaffl, 2006).

3.2.7.3 **Reverse transcription**

For Real- Time PCR analysis, 1.5 µg of mRNA were reverse transcribed into single stranded complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) following manufacturer´s instruction (Table 5.2) (Wang *et al.*, 2012). The reverse transcription polymerase chain reaction allows the qualitative and quantitative assessment of gene expression by creating cDNA. The CAS Robotics 1200 (Qiagen, Germany) pipetted the reaction samples. Its six station channel and liquid level sensor assures reliability and accuracy. Reverse transcription was achieved on the S1000^{TM} Thermal cycler (BioRad, UK) in a reaction volume of 30 µl. Following thermal conditions were used: initially 10 min at 25 °C for annealing of the random primer at the RNA, followed by extension of the complementary strand for 120 min at 37 °C and finalized by 5 min at 85 °C to inactivate the reverse transcriptase. To protect the new synthesized cDNA from DNases, the cDNA was resuspended in 200 µl 0.5 % TE Buffer pH 7.4.

3.2.7.4 **Primer design**

For real-time qPCR analysis, specific primer of each target gene were designed based on previous studies (Apte & Daniel, 2009, Burpo, 2001, Chomczynski, 1993, Fleige & Pfaffl, 2006) as well as with the help of more than three online tools (Birney *et al.*, 2004, Flicek *et al.*, 2012, Pruitt *et al.*, 2009, Zuker, 2003): the "Ensembl Genome" browser for sequence annotation, "Primer Blast" provided by the National Center for Biotechnology Information (NCBI) for primer design and the "Mfold Web Sever" for analysing possible secondary structures.

First, with the help of the "Ensembl Genome" browser the right target sequence for the primer design was chosen, taking into account the different splice variants of each gene. The "Ensembl Genome" browser is supported by several institutes: the European Bioinformatics Institute (EBI), the European Molecular Biology Laboratory (EMBL), the Wellcome Trust Sanger Institute (WTSI) and the Wellcome Trust Genome Campus

(http://www.ensembl.org/index.html).

Next, the online "Primer-BLAST" tool was used to design primers of a target region of each gene of interest (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). For the calculation of the annealing temperature, thermodynamic calculations of Santa Lucia 1998 were used (SantaLucia, 1998).

To prove the efficacy of the designed primer pairs by in-silico prediction of possible secondary structures in the amplicon, their sequence was analysed in a FASTA-format using the "mfold" algorithm at "The mfold Web" server provided by the RNA Institute College of Arts and Sciences in US (http://mfold.rit.albany.edu/?q=mfold) (Zuker, 2003). Primer optimization strategies were performed based on previous studies (Apte & Daniel, 2009, Bustin *et al.*, 2005, Fleige & Pfaffl, 2006, Pfaffl, 2001, Wang & Seed, 2003). The sequences and the estimated size of all primers used to amplify the genes of interest are listed in Figure 4.

3.2.7.5 **Semi-quantitative PCR**

To identify the expression of the gene of interests in the target cells, PCR was performed, followed by electrophoresis.

Before the newly designed primer could be used for further PCR investigations, the primer had to be proven to recognize and bind only the target gene of interest and furthermore to amplify the correct template. Therefore, evaluation of the expected template and amplified products in expected size was performed by semi-quantitative PCR, proven and visualized by gel electrophoresis. For better accuracy the melting temperature (Tm) might had to be optimized or the primer had been re-designed until accurate specific primer templates were proven.

PCR is a method developed by Kary Mullis in the 1980s (Mullis *et al.*, 1986) and optimized in last years. PCR was done according to manufacturer´s instruction (Table 6) and previous studies (Lorenz, 2012). cDNA of cells, known to express the gene of interest were amplified by PCR reaction in the S1000^{TM} Thermal cycler (BioRad, UK) in a 20 µl reaction volume containing 0.2 µM/ primer (forward/ reverse), 200 µM dNTPs (PROMEGA GmbH, Germany), 2.5 µl Loading Dye (5-Prime GmbH, Germany) and 0.125 µl Perfect Taq Plus polymerase (5-Prime GmbH, Germany). Thermal condition included an initial step to activate the Taq polymerase at 95 °C for 3 min, followed by 35 cycles of 3 steps: 30 s at 94 °C (denaturation), 30 s at 60 °C (annealing temperature varied for certain amplicons depending on the primer melting temperature) and 30 s at

72 °C (complementary elongation of the templates by the polymerase). Finally, an extension or elongation step for 10 min at 72 °C was performed to ensure that no single-stranded DNA is remaining.

3.2.7.6 **Gel electrophoresis**

To detect and identify the amplified PCR products of the genes of interest, DNA gel electrophoresis was realized (Mavre *et al.*, 2010). Gel electrophoresis is a common technique to detect and identify DNA fragments based on the size (Mavre *et al.*, 2010). Agarose was dissolved in TBE-buffer (Tris-Borate-EDTA-buffer) to a concentration of 1 % agarose solution. Next, 2.5 ul/ 100 ml ethidium bromide (Sigma Aldrich Chemie GmbH, Germany) was added to the solution. The agarose gel was cast in a gel tank, any bubbles were removed by a sterile pipette tip, the gel comb was added and the gel allowed to set (Kean, 2006). The agarose gel (1 %) was added into a horizontal tank containing TBE-buffer. A 100 bp DNA Ladder was used as a DNA standard marker for the evaluation of the template sizes. 17 µl (= 37.5 ng) of each amplified cDNA product were carefully inserted in the already preformed corned gel chambers. Gel electrophoresis was carried out at an electrical field of 120 V. The cDNA, which is negatively charged by comprised phosphates, migrates and separates based on their size towards the anode, after applying the electrical field of 120 V. Distributed amplicons were visual verified by an UV-Transluminator to confirm the expected size and the lack of unspecific PCR products.

3.2.7.7 **Real-time quantitative PCR (RT-qPCR)**

The real-time qPCR, a sensitive and precise method to quantify the relative expression of each gene of interest (Derveaux *et al.*, 2010), was performed with the Corbett Rotor Gene 6000 (Qiagen, Germany). Each sample was analysed in duplicates in a total reaction volume of 20 µl containing 4 µl of cDNA and 16 µl of Master Mix. The 16µl Master Mix was composed as follows: 10 µl of SensiMix SYBR NO-ROX Kit (Bioline, UK), 0.4 µl (10 pmol/ ul) of each forward/ reverse primer and 5.2 µl of nuclease-free water. Pipetting of the RT-qPCR reactions was realised with the CAS 1200 Robotics (Qiagen, Germany) to ensure maximum accuracy and replicability. Each run started with 95 °C for 10 min to activate the Hot- Start DNA polymerase, followed by 40 cycles of following conditions: 95 °C for 15 seconds for denaturation, 60 °C for 20 seconds for annealing of the primers, and finally 72 °C for 20 seconds for complementary

elongation. Primer specificity of each target gene was examined by the melt curve analysis and the size verification by gel electrophoresis (Bustin *et al.*, 2005, Wang & Seed, 2003). The efficiency (E) of the primers were evaluated by a standard curve based on a serial dilution of the target gene containing cDNA (3 ng; 1.5 ng; 0.75 ng; 0.375 ng) (Kubista *et al.*, 2006). The measured cycle threshold values (Ct) were plotted against fluorescence in a diagram and analysed using the equation: $E= (10^{(-1/slope)})-1$ (Kubista *et al.*, 2006) with the help of the Rotor Gene 6000 Software (Qiagen, Germany). All designed primers (Table 7.2/ Fig. 4) demonstrated an efficiency of 1, except the primer of MKP-1 = 0.87. At the end of each run of the RT-qPCR, a melt curve program followed by continuous fluorescence measurement under following conditions: increasing heating rate of 1 °C per 5 seconds from 72°C to 95°C. The melt curve characterizes the specific chemical bond-properties of each amplicon, examined by increasing temperature leading to denaturation of the dsDNA. Denaturation depends on the length and sequence of the amplicons. The melting point is a specific point of temperature where half of the dsDNA already separates in single DNA strands. The SensiMix SYBR NO-ROX Kit contains a SYBR Green-intercalating dye. The dye gets excited and fluoresces when intercalated into dsDNA. The fluorescence emission of SYBR-green can be measured at a wavelength between 450-510 nm. During the melt curve analysis, fluorescence of the intercalated SYBR dye decreases, caused by the loss of helical structure of the DNA. The continuous fluorescence measurement plots derivative fluorescence (dF/ dT) versus temperature into a diagram. At a specific temperature, the derivative fluorescence increases to a sharp slope, defined as the melting point. The presence of unintended PCR-products or primer dimers can be detected by the occurrence of additional peaks in the melt curve analysis, leading into further primer-design or PCR optimization by increasing Tm or changing the amount of magnesium in the Master Mix. A RT-negative sample was used as negative control to detect the presence or absence of contaminating genomic DNA. In the final experiments no genomic DNA or primer dimers were detected. Analysis of the relative expression in RT-qPCR was performed as follows:

In PCR, ideally the amount of amplicons doubles each cycle, leading to exponential amplification. In RT- qPCR, a step to analyse newly amplified dsDNA by detection of fluorescent intercalating SYBR-Green dye was inserted after each cycle. Measured data were plotted into a diagram depicting the fluorescence versus the temperature (Fig. 7). To analyse the amount of amplified products in relation between treated and untreated

Fig. 7: Fluorescence of amplified RT-qPCR products, investigating the expression of IL6 in different conditions

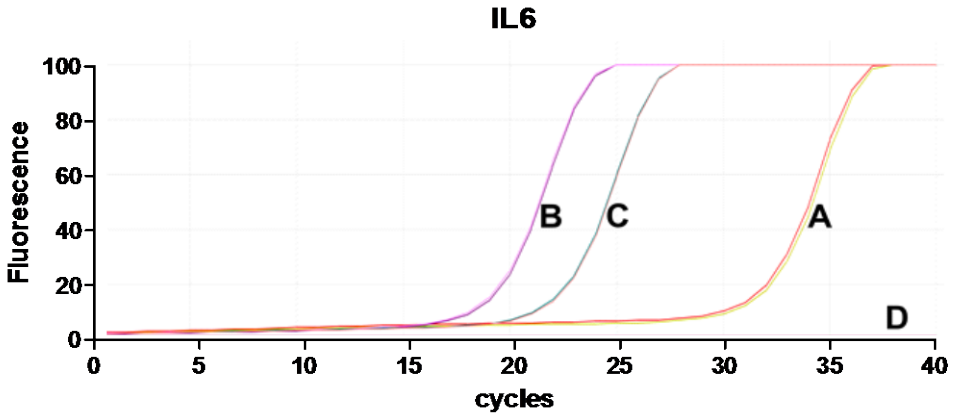

Fig. 7: The figure shows a RT-qPCR plot determining changes in IL6-expression by investigating different samples: (A) Control, (B) *C. albicans* (UV) ; (C) *C. albicans* (UV) + atRA; (D) RT-negative. The diagram shows the cycles versus fluorescence of the cDNA for different conditions. RT-qPCR was performed by the Corbett Rotor Gene 6000.

samples of equal conditions, differences in Ct-values were documented for further analyses after setting a cycle-threshold (Ct). The cycle-threshold, defines the cycle number where the signal of SYBR-Green fluorescence reaches exponential phase. Which is usually set at the exponential phase of the curve. The relative expression of the target genes was analysed using a modified Pfaffl method (Derveaux et al., 2010, Pfaffl, 2001, Rieu & Powers, 2009). To determine significant differences in the mRNA expression between different experimental conditions, the relative quantity for each sample was calculated using following equation: $E=10^{(-1/ct)}$, here E is the efficiency and ct the threshold cycle (Pfaffl, 2001). The relative quantity for each sample was then normalized to the geometric mean of two house-keeping genes in this experiment: hypoxanthine phosphoribosyltransferase1 (HPRT1) and peptidylpropyl isomerase B (PPIB) (see table 7.2 and figure 4). Reference genes (or housekeeping genes) are stably expressed and should not change their expression among the experimental conditions tested. The stability of the housekeeping genes was assessed using the BestKeeper algorithm (Pfaffl et al., 2004). The normalised relative quantity values were log2-transformed for further statistical analysis with GraphPad Prism 5.0. All analysed sample groups were either analysed in the same run in duplicates or calibrated by an internal control in duplicates.

3.2.8 **Flow cytometry**

To analyse the atRA-induced changes of Dectin-1 and TLR2 expression on the surface of the monocytes flow cytometry was used (Herzenberg *et al.*, 2002).

In flow cytometry, fluorochrome coupled antibodies are measured by lasers and detectors, detecting the fluorochrome characteristic emission wavelength (Baumgarth & Roederer, 2000, Herzenberg *et al.*, 2002). In our study, FITC- coupled antibodies and APC-coupled antibodies were used (FITC ~ 525 nm; APC~ 660 nm). Light scatter analysis gives additional information about granulation and size of the cells. For flow cytometry analysis, monocytes were harvested after 12 hours, 18 hours and 24 hours of stimulation. Viability of the monocytes was ≥ 90%, as evaluated by trypan blue staining and propidium iodide 1.0 mg/ ml (VWR International, Germany). The cells were harvested and washed with cold flow cytometry buffer (DPBS containing 2 % FCS), filled up to an amount of 1.5 * 10^6 cells/ 400 µl buffer and added in 96- well plates by an amount of 3.75 * 10^5 cells/ 100 µl buffer/ well (Jouault *et al.*, 2006).

Cells of each stimulation case were stained with either Dectin-1 antibodies or TLR2 antibodies separately (Table 9). Antibodies are conjugated with a fluorescent marker for measurement and visual verification. Cells stained with Anti-Dectin-1 antibody were conjugated with APC labelled goat anti-mouse antibody in a secondary step, whereas the Anti-TLR2 antibody was already FITC conjugated, so no secondary step was needed (Table 9) (Ambarus *et al.*, 2012, Elsori *et al.*, 2011, Li *et al.*, 2012).

Each staining step took about 30 min of incubation time, followed by several washing steps and further incubation periods with flow cytometry buffer. All cells went through all washing steps and incubation periods, incubated with either antibodies or flow cytometry buffer to receive results under equal conditions. Preliminary experiments using monocytic THP-1 cells and monocytes gave information about the adequate concentration of the antibodies, fluorochrome and isotypes according to the size of the cell population (Hulspas *et al.*, 2009, Tung *et al.*, 2007). Unstained samples were used as negative control to take into account the auto fluorescence of the cells (Hulspas *et al.*, 2009). Specificity of the antibodies was verified by isotype control samples (Hulspas *et al.*, 2009, Tung *et al.*, 2007). Samples were measured in a FACS ARIA II machine (BD Biosciences, Germany). The flow cytometry data were analysed according to previous studies with the help of the FlowJo 7.6.4. Software (Herzenberg *et al.*, 2006, Tung *et al.*, 2007). The mean of the fluorescence intensity (MFI) of each case was normalized to the MFI of the corresponding unstained sample (Dendrou *et al.*, 2009). The relative

mean fluorescence intensity of the TLR2 and Dectin-1 of four independent experiments was analysed, each sample verified in triplicates.

3.2.9 Cytokine measurement

To quantify the amount of secreted cytokines by monocytes after challenging with UV-inactivated *C. albicans* yeasts for 16 hours, the supernatant of the cell culture was analysed with the use of commercially available ELISA-Kits: TNFα, IL6, IL12b, IFN-β and IL10 (Table 12). The concentration of each of the secreted cytokines of different conditions of five independent experiments was analysed, each sample verified in triplicates. For quantitative enzyme-linked immunosorbent assay (ELISA) a capture antibody of the target cytokine was pre-coated on Maxisorp F 96-well plates (VWR International, Germany) based on manufacturer´s instruction (Table 12). After a washing step, an equal amount of each supernatant was added to each well. The ideal dilution of the supernatant was used in each case and all samples were analysed in triplicates. Furthermore, a positive control with a known concentration and its serial dilution was included for further data analysis. The diluent was used as a negative control and for background correction. After an incubation period of the supernatant based on manufacturer´s instructions several washing steps followed. Same procedure was always repeated after adding a new module of the ELISA Kit. The next module of the ELISA Kit was the addition of the detection antibody, followed by the sensitive enzyme linked with a secondary antibody in a following step. This secondary antibody connects to the detection antibody. In one of the last steps, the substrate was added. The substrate is degraded into fluorescent products by the already added and linked enzyme. The higher the amount of cytokines in a well, the more quantity of linked enzyme will be in a well, degrading the substrate. Consequently, more fluorescent products are present. Before measuring the optical density (OD) at indicated wavelength in the Infinite M200 reader (Tecan, UK), a stop solution was transferred to each plate to prevent eventual saturation of the signal. The concentration of cytokines was calculated in each case with the help of the analogue standard curve by five-parameter logistic analysis using Magellan v.6 Software (Tecan, UK) (Findlay & Dillard, 2007).

3.2.10 Statistical analysis

Statistical analysis was performed using the Graph Pad version 5 software (San Diego, US). One-way ANOVA (Vanoirbeek *et al.*, 2013) with Dunnett´s post-hoc test or two-

sided t-test were used for statistical analysis. For flow cytometry, data were assessed by the Page´s L Test.

The level of statistical significance was set at * $p \leq 0.05$; ** $p<0.01$; *** $p<0.001$.

4. Results

4.1 AtRA is able to modulate the *C. albicans*-induced cytokine production in human monocytes

To ascertain the modulatory effect of atRA on the immune response induced by *C. albicans*, monocytes facing UV-inactivated yeast in presence or absence of 1 µM atRA were analysed. In a first assay, alteration in the expression of the pro-inflammatory cytokines TNFα, IL6, IL12b and the anti-inflammatory cytokine IL10 was analysed at transcriptional level by Real-Time qPCR.

After 5 hours of incubation with UV-inactivated *C. albicans* yeasts, we could observe a significant increase of all four cytokines on mRNA level (Fig. 8A). However, in the presence of atRA in the cell culture medium a significant suppression of the pro – inflammatory cytokines occurred. We observed a significant downregulation of TNFα from a 78-fold mRNA expression upon stimulation with *C. albicans*, to a 21-fold mRNA expression by the influence of atRA. Equal results were observed for the other two pro-inflammatory cytokines IL6 and IL12b. The presence of atRA led to a significant downregulation of up to 94 % and 97.9 % of the *C. albicans*-induced mRNA expression, respectively. This inhibitory effect of atRA on all three pro-inflammatory cytokines seem to occur in a dose-dependent manner, already observed in very low concentration like 0.01 µM atRA (Fig. 8B).

The expression of the anti-inflammatory cytokine IL10 was clearly increased by the stimulation with C. *albicans*. The same response was also observed for addition of atRA alone (data not shown). Nevertheless, the presence of atRA in the culture medium while monocytes were challenged with UV-inactivated *C. albicans* yeasts showed no effect at transcriptional level. However, all-trans retinoic acid seems to modulate the expression of the pro-inflammatory cytokines TNFα, IL12b, IL6 in the *C. albicans* infection.

Next, we investigated the impact of atRA on the protein level of these pro-inflammatory cytokines and the anti-inflammatory cytokine IL10 in *C. albicans* infection. We measured the level of the cytokines TNFα, IL6, IL12b and IL10 in the supernatant of the cell culture, secreted by monocytes after 16 hours of fungal stimulation alone or in presence of 1 µM atRA (Fig. 9) with the use of specific ELISA-Kits.

Upon *C. albicans* infection, a clear increase in all secreted pro-inflammatory cytokines could be seen. In the presence of 1 µM atRA, we observed a significant downregulation of TNFα by a mean of 72 %, IL6 by a mean of 68.1 % and IL12b by a mean of 84.4 %.

In contrast, addition of atRA to the culture medium did not induce a change in protein level of the anti-inflammatory cytokine IL10.

Summarizing, all-trans retinoic acid seems to modulate the expression of the pro-inflammatory cytokines TNFα, IL12b, IL6 in the *C. albicans* infection. Moreover, atRA seems to be an anti-inflammatory modulator in monocytes upon *C. albicans* infection. Next, we investigated whether atRA exerts its modulatory role upon cytokine expression by the modulation of PRRs like Dectin-1.

Fig.8: Modulatory effect of atRA on monocytes upon cytokine expression in the presence of *C. albicans* for 5 hours

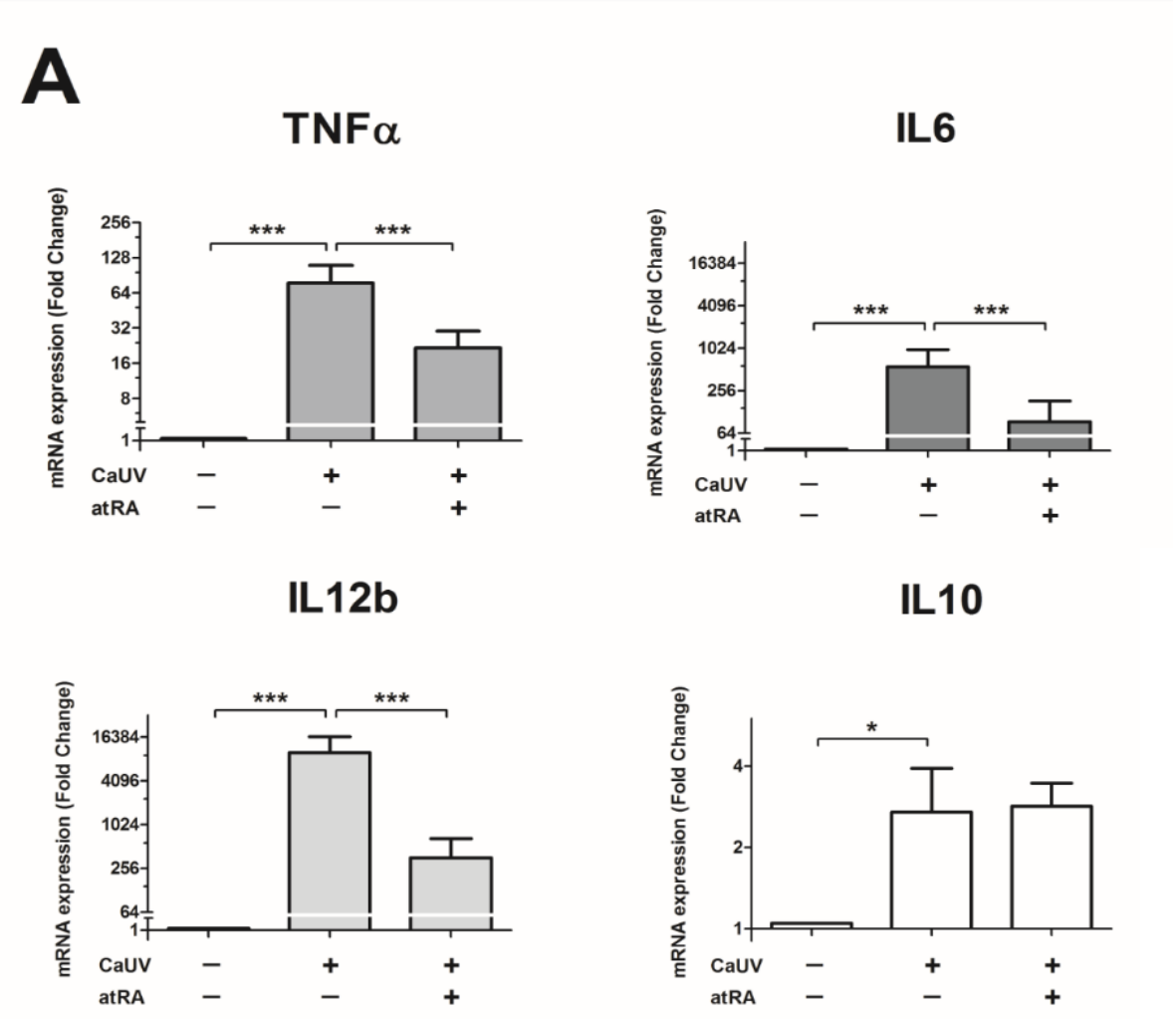

Fig. 8A: The graphs show the *C. albicans*-induced mRNA expression of the TNFα, IL6, IL12b and IL10 in the presence or absence of 1 µM atRA. Results are shown as mean fold expression +/- SEM of five independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 8B: Chart describes the inhibitory impact of different concentrations of atRA (from 0.01 µM to 10 µM) on the pro-inflammatory cytokine mRNA level upon *C. albicans* infection.

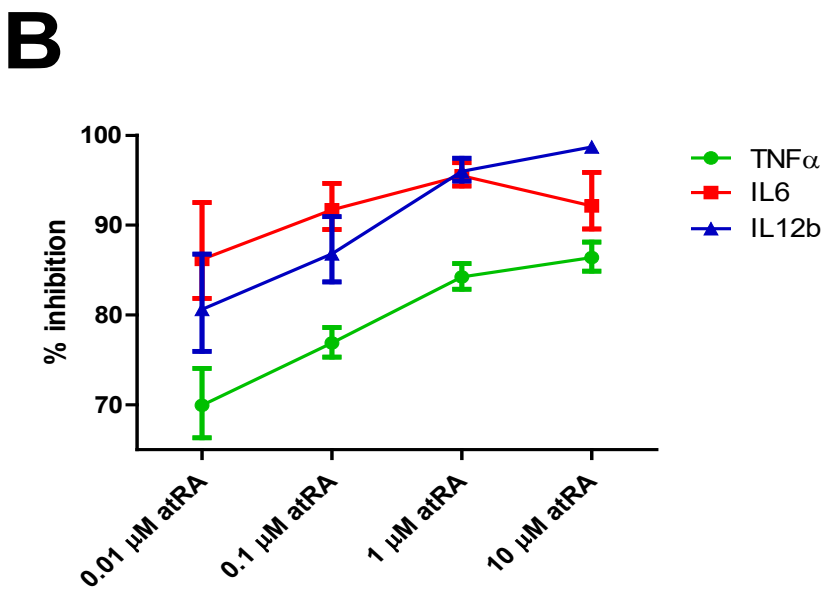

Fig 9: Modulatory role of atRA on the secreted cytokines by monocytes in fungal infection

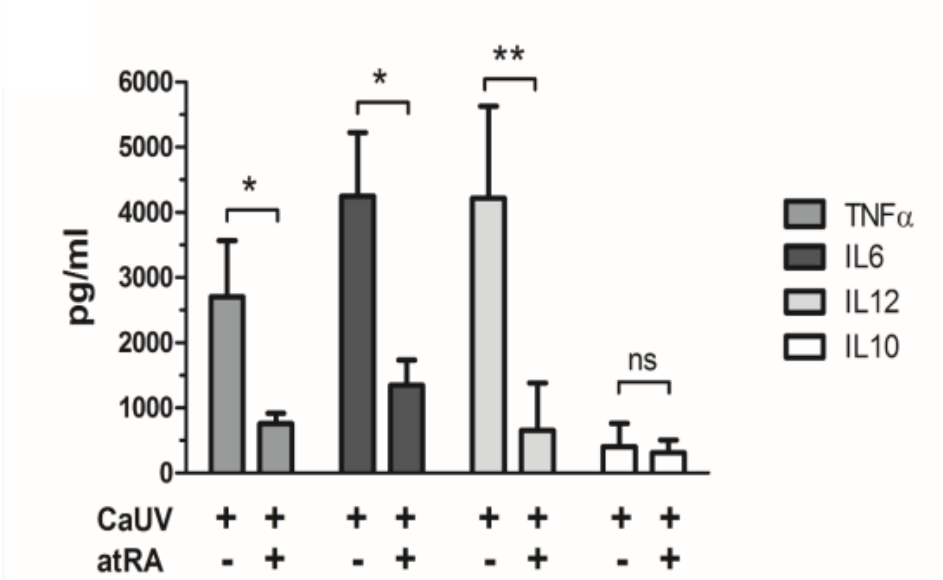

Fig. 9: For 16 hours, human monocytes were challenged with UV-inactivated *C. albicans* yeast in the presence or absence of 1 µM atRA. Supernatants of five independent experiments were collected and examined for variances in cytokine release with the use of specific ELISA Kits. Results are shown as mean concentration (pg/ ml) +/- SEM.

* $p<0.05$; ** $p<0.01$; ns not significant

4.2 AtRA modulates the Dectin-1-mediated cytokine production in human monocytes

To address specifically the effect of atRA on the Dectin-1-mediated response, we used beads coated with β-1.3 glucan as pro-inflammatory trigger. These β-1.3 glucan beads mimic the shape of *C. albicans* yeast, but inducing a specific Dectin-1 response.

Human monocytes were incubated with β-1.3 glucan beads for 5 hours and 16 hours in the presence or absence of 1 μM atRA, monitored by microscopy.

As shown in figure 10, we could observe the attachment of the beta-glucan beads to the monocytes after 5 hours of incubation (Fig. 10). At this time point, we measured the expression of cytokines at transcriptional level by RT-qPCR. Monocytes treated with β-1.3 glucan beads showed a significantly increase in the gene expression of the pro-inflammatory cytokines TNFα, IL6 and IL12b. However, the presence of atRA led to a clear downregulation of the specific Dectin-1-mediated cytokine expression of all pro-inflammatory cytokines: near to the initial point by the inhibition of 73 % and 86.6 % for TNFα and IL12b respectively and in the case of IL6 an inhibition up to 65.2 % (Fig. 11A).

Fig 10: Monocytes treated with β-1.3 glucan coated beads in presence or absence of atRA monitored under microscope at several time points.

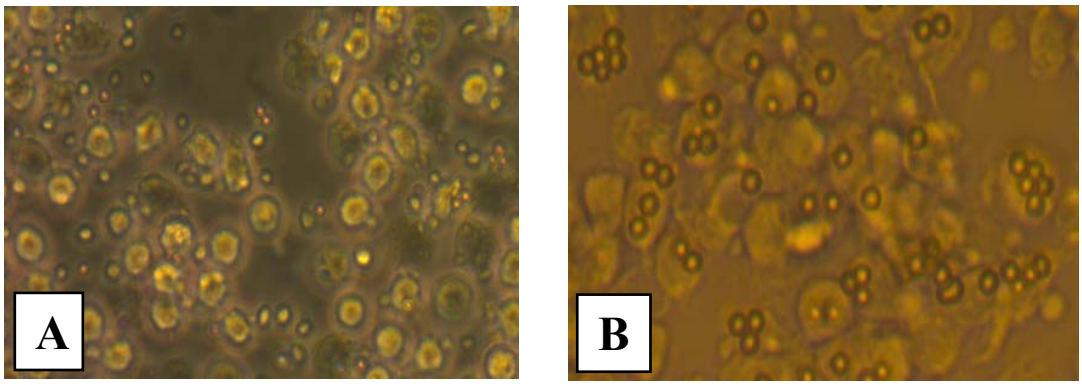

Fig. 10A-B: The images show monocytes treated with β-1.3 glucan coated beads in the presence of atRA at zero hour (A) and five hours (B) of incubation. Monocytes were monitored under microscope at several times.

In the next step, we investigated the effect of atRA at the protein level in the Dectin-1-mediated cytokine response. We measured the level of the secreted cytokines TNFα, IL6, IL12b and IL10 in the supernatant of the cell culture with the use of specific ELISA-Kits after 16 hours of stimulation in the same experimental conditions (Fig. 11B). We observed a clear downregulation of the β-1.3 glucan triggered cytokine release in the presence of

atRA by an inhibition of 88.4 %, 92.2 % and 37.4 % for TNFα, IL6 and IL12b respectively. Furthermore, we investigated the impact of atRA at the anti-inflammatory cytokine IL10 in the Dectin-1-mediated response. In the presence of atRA, we observed a significant upregulation of the already enhanced IL10 cytokine release by the β-1.3 glucan beads (Fig. 11).

These findings show clearly the anti-inflammatory impact of atRA on the specific Dectin-1-induced immune response at transcriptional and post-translational level. Since we could observe a modulation of the Dectin-1 function in terms of receptor-dependent cytokine production, we next investigated whether atRA is able to regulate the expression of this pattern recognition receptor itself.

4.3 AtRA modulates the expression of relevant PRRs

4.3.1 The expression of Dectin-1 is modulated by atRA

We investigated the variation in Dectin-1 mRNA expression in monocytes upon *C. albicans* infection in presence or absence of atRA by RT-qPCR after 5 hours and 16 hours of stimulation. Our results show that the addition of atRA led to a significant reduction of the Dectin-1 expression in human monocytes upon *C. albicans* infection after 5 hours and 16 hours of stimulation. Interestingly, we could also observe that *C. albicans* alone was able to downregulate its recognizing pattern recognition receptor in a significant manner (Fig. 12A). To confirm these findings at protein level, the impact of atRA on Dectin-1 expression upon *C. albicans* infection was determined by flow cytometry over a one-day period: examinations were done after 12 hours, 18 hours and 24 hours of stimulation (Fig. 12B-C). As shown in figure 12B, we could observe a vitamin A-mediated drop of the expression of Dectin-1 on the cell surface of the human monocytes at protein level. When we analysed the inhibitory effect of atRA on the Dectin-1 receptor expression over time, we could observe an increasing inhibitory effect, with a maximum at 24 hours of stimulation, confirming the results at transcriptional level. The inhibitory effect of atRA on the Dectin-1 expression could also be observed in the absence of inflammatory trigger. Stimulation of monocytes with 1 µM atRA alone lead to a downregulation of the receptor, similar to the one observed in the infection model. This effect could be confirmed at both transcriptional and protein level (Fig. 13). Since atRA is known to play a key role in differentiation processes, we investigated whether the observed modulatory effect of atRA on the Dectin-1 expression might be due to the

differentiation of monocytes into dendritic cells (DCs) or macrophages. However, monocytes terminally differentiated into monocytic-derived DCs by stimulation with GM-CSF and IL-4 showed a comparable impact of atRA on the Dectin-1 expression (Fig. 14)

Fig. 11: Modulatory effect of atRA on the Dectin-1-mediated cytokine expression and secretion

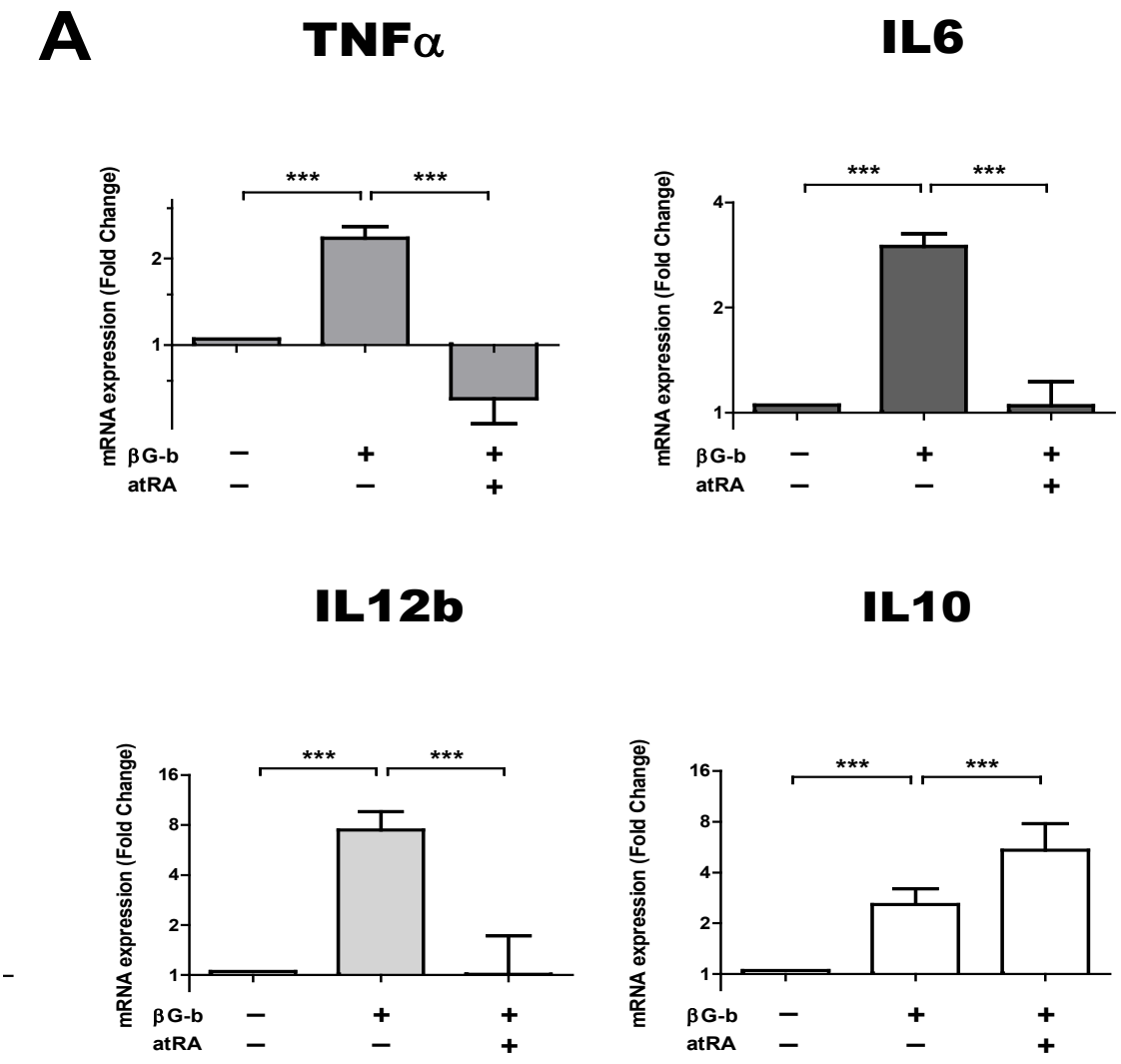

Fig. 11A-B: Monocytes were stimulated with β-1.3 glucan coated beads for 5 hours (A) and 16 hours (B) to assess the impact of atRA on the Dectin-1-mediated cytokine production.

Fig. 11A: Monocytes were challenged with β-1.3 glucan beads in the presence or absence of 1 µM atRA for 5 hours. All Results are shown as mean fold change +/- SEM of five independent experiments.

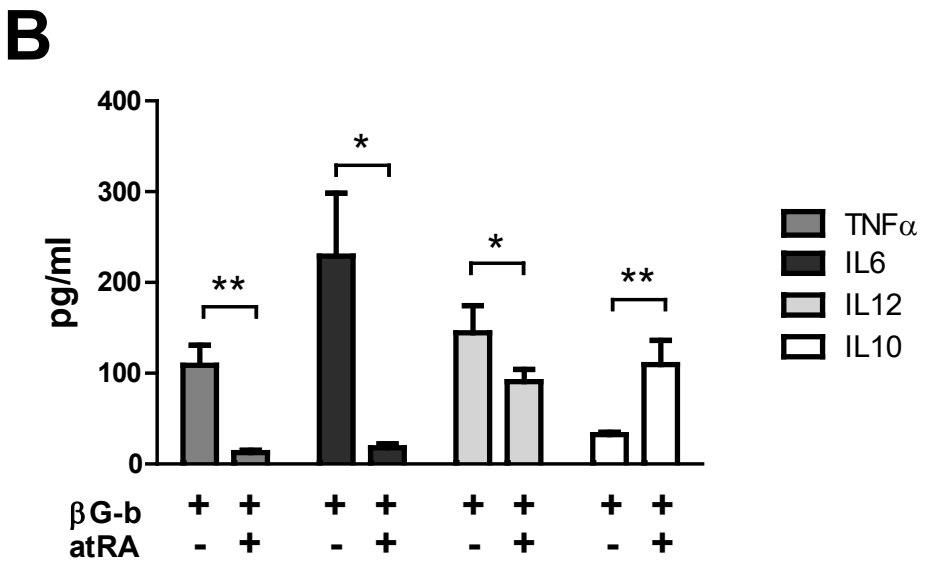

Fig. 11B: Measurement of the secreted cytokines after 16 hours of stimulation, expressed as mean concentration (pg/ ml) +/- SEM of five independent experiments.
Fig. 11A-B: * p<0.05; ** p<0.01; *** p<0.001

Fig. 12: Modulatory role of atRA on the expression of the Dectin-1 receptor in human monocytes at transcriptional (A) and protein level (B-C) in fungal infection

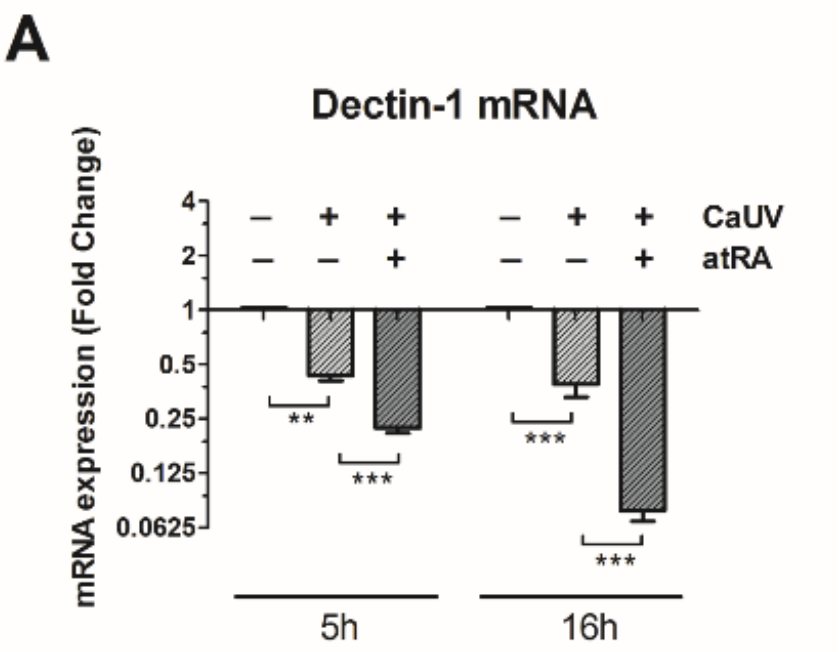

Fig. 12A: The figure shows the mean mRNA expression +/- SEM of the Dectin-1 receptor of five independent experiments in presence or absence of 1 μM atRA in fungal infection after 5 hours and 16 hours of stimulation. **p<0.01; ***p<0.001

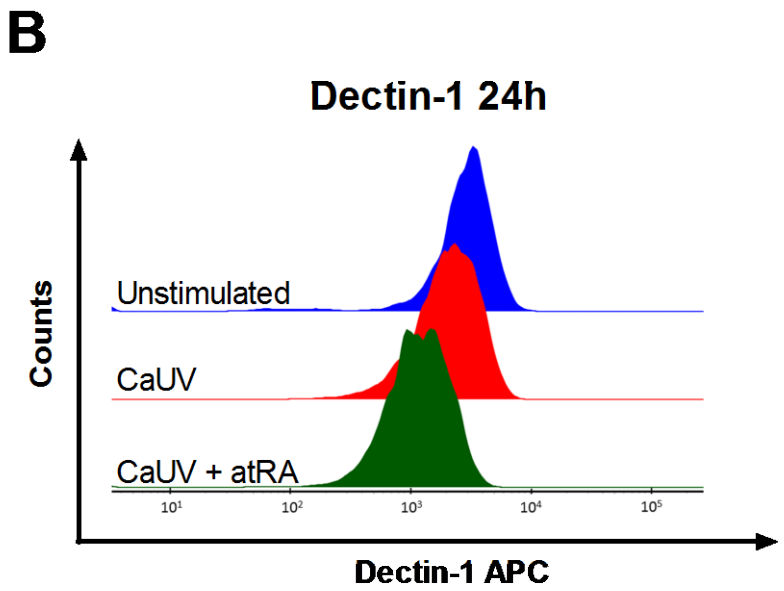

Fig. 12B: The figure shows a flow cytometry plot of Dectin-1 APC after 24 hours of stimulation with either *C. albicans* or *C. albicans* and 1 µM atRA.

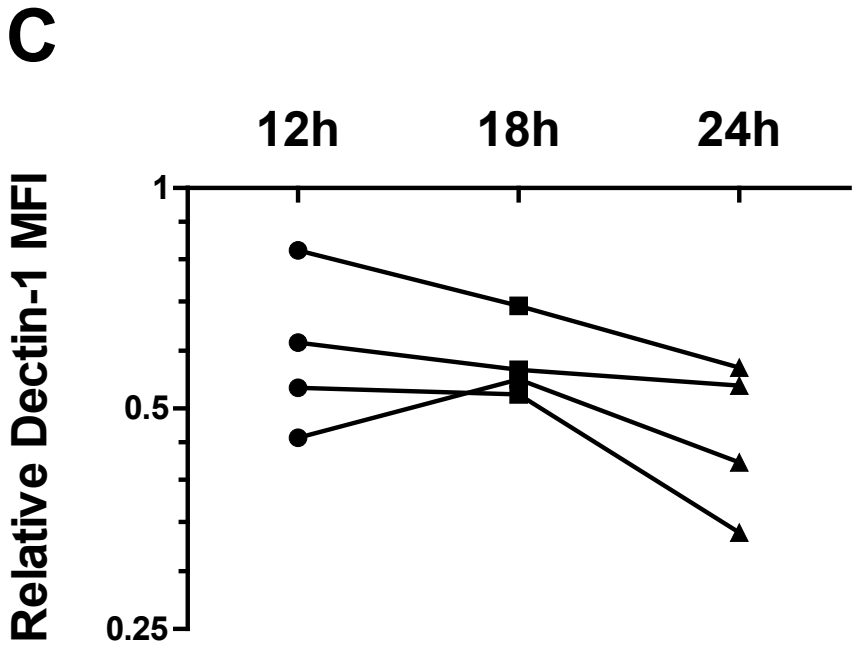

Fig. 12C: The diagram shows the relative mean fluorescence intensity (MFI) of Dectin-1 over a one-day period. The relative MFI is defined as the MFI of the monocytes stimulated by *C. albicans* and 1 µM atRA divided by the MFI of the monocytes challenged only with UV-inactivated *C. albicans* yeast.

Fig. 13: Modulatory effect of atRA alone on the transcriptional and post-translational expression of the PRR Dectin-1

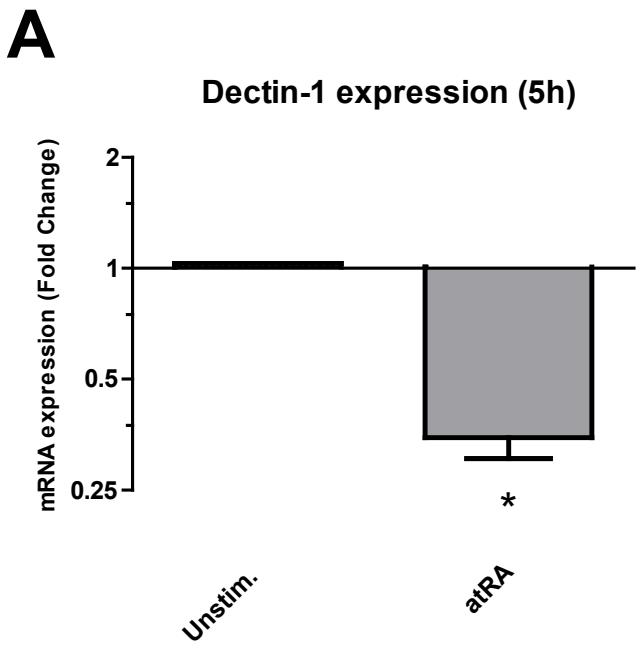

Fig. 13A: The mRNA expression of Dectin-1 after 5 hours of stimulation with 1 µM atRA. Results are shown as mean fold expression of three independent experiments. For statistical analysis t-Test was used**.** * $p<0.05$

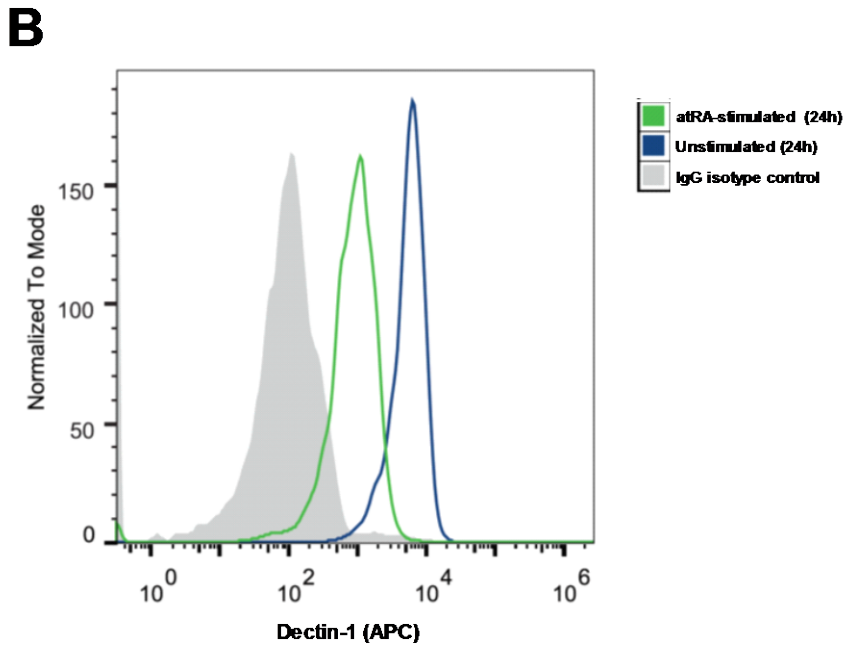

Fig. 13B: Dectin-1 expression on the cell surface of human monocytes was analyzed by flow cytometry after 24 hours of incubation with 1 µM atRA.

Fig. 14: Modulatory impact of atRA on the Dectin-1 expression in monocytic-derived dendritic cells

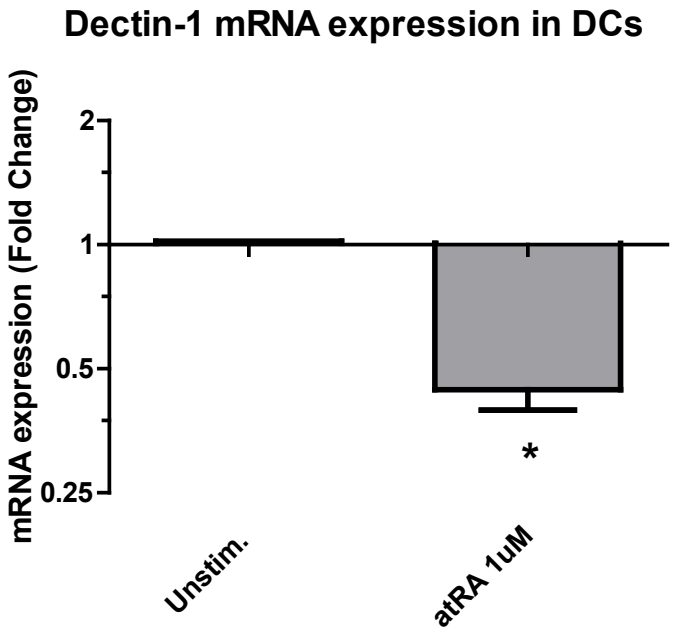

Fig. 14: Human monocytes were stimulated with GM-CSF and IL-4 for one week. Terminally differentiated monocytic-derived DCs were stimulated with 1 μM atRA for 5 hours before transcriptional analysis of Dectin-1. Analysis was performed in three independent experiments.
* $p<0.05$

4.3.2 Impact of atRA on the expression of Dectin-1 co-receptors

Since several receptors are known to cooperate with Dectin-1, leading to an amplification of a specific and effective host defence, we next investigated whether atRA could also have an effect on the expression of the known co-receptors of Dectin-1:TLR2 and Gal-3. As shown in figure 15, we observed an influence of both atRA and *C. albicans* on the mRNA expression of these two PRRs on monocytes after stimulation periods of 5 hours and 16 hours. At both times, we observed that *C. albicans* lead to an upregulation of the expression of TLR2 and Gal-3. Nevertheless, when atRA was added, we observed a significant downregulation.

4.3.3 Early regulation of PRRs by atRA

Our results show that the mRNA expression of both Dectin-1 and its co-receptors are downregulated in the presence of atRA. This raised the question whether the drop in cytokine expression could be attributed to the downregulation of these receptors.

To investigate further this causality, we next investigated the impact of atRA on the expression of the receptors at earlier time points. We investigated the expression of the surface proteins Dectin-1 and TLR2 by flow cytometry and Gal-3 by western blot after 4 hours of stimulation with *C. albicans* in presence or absence of 1 µM atRA. Although we found no variation in the post-translational expression of TLR2 and Gal-3, an incipient decrease in the Dectin-1 expression on the cell surface could already been observed after 4 hours, where both atRA and *C. albicans* were able to downregulate the expression of the Dectin-1 receptor (Fig 16A-C).

Fig. 15: Modulatory impact of atRA on the mRNA expression of TLR2 and Gal-3 upon *C. albicans* infection

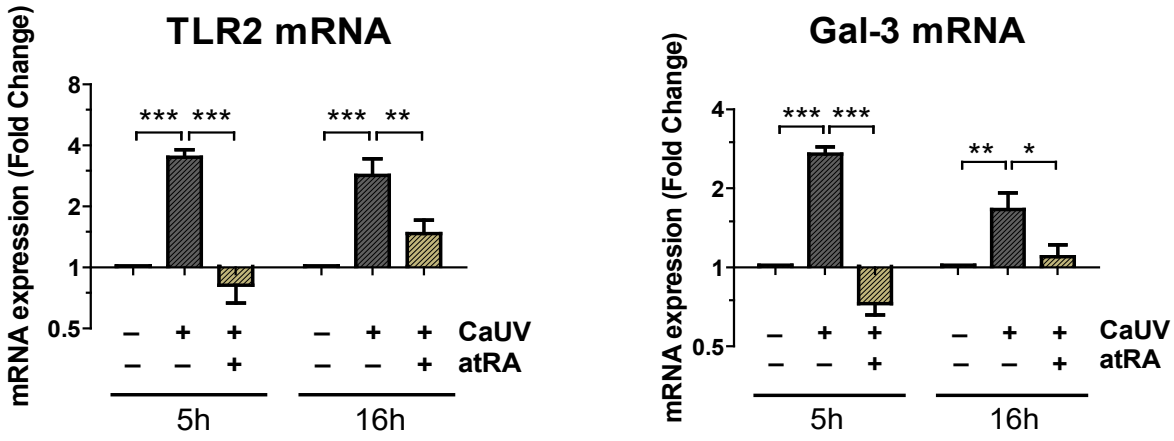

Fig. 15: Both figures show the mean relative mRNA expression +/- SEM in monocytes of five independent experiments after 5 hours and 16 hours of incubation with *C. albicans* in presence or absence of 1 µM atRA. *p<0.05; **p<0.01; ***p<0.001

4.3.4 Short versus long atRA pre-treatment

We have observed that the effect of atRA on Dectin-1 expression in fungal infection is highest after 24 hours of stimulation. Therefore, we compared the inhibitory power of atRA on larger pre-incubation periods of 24 hours as compared to only 0.5 hours.

Surprisingly, a longer pre-treatment of atRA in monocytes up to 24 hours led to a reduced effect in cytokine inhibition (Fig. 17). Whereas atRA showed a slight reduction on the inhibitory effect of 9.3 % and 16.52 % on the expression of TNFα and IL12b respectively. The impact on the IL6 expression was reduced up to 63.3 % after 24 hours of stimulation. According to our data, atRA seems to show its highest anti-inflammatory effect at transcriptional level after a short period of pre-stimulation, suggesting a causal direct

molecular mechanism.

Fig. 16: Post-translational modification on the PRRs Dectin-1, TLR2 and Gal-3 in monocytes by atRA at an early stage of the *C. albicans*-induced inflammation.

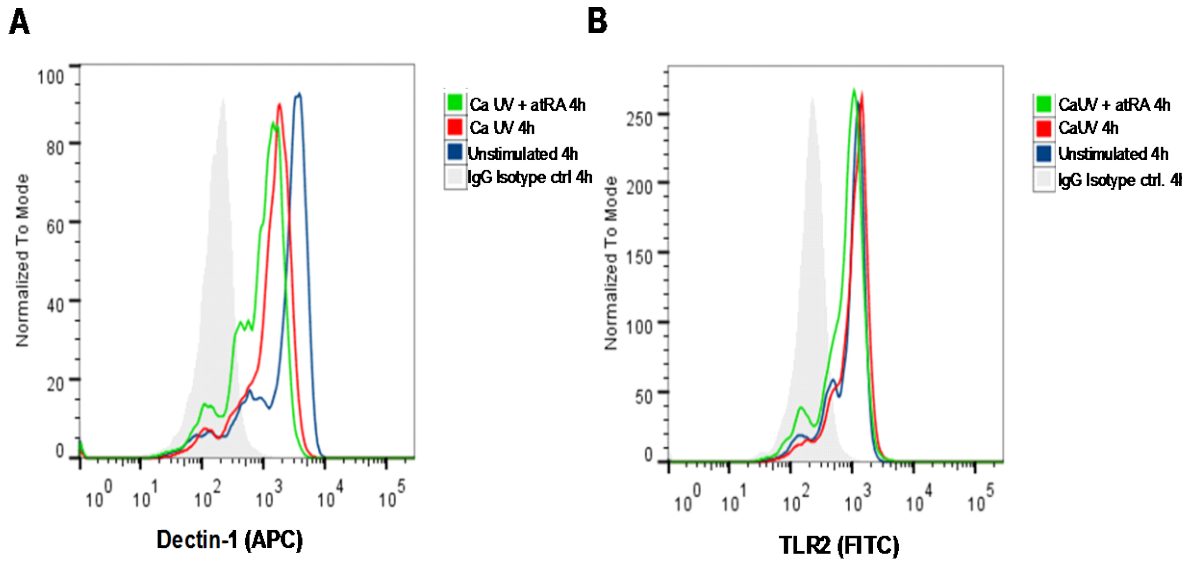

Fig 16A-C: Modification of the expression levels of the PRRs Dectin-1, TLR2 and Gal-3 in monocytes by 1 µM atRA upon *C. albicans* infection were investigated either by flow cytometry (9A-B) or by western blot (9C) after 4 hours of incubation. Three independent experiments were realized.

Fig. 16A-B: Representative results of flow cytometry.

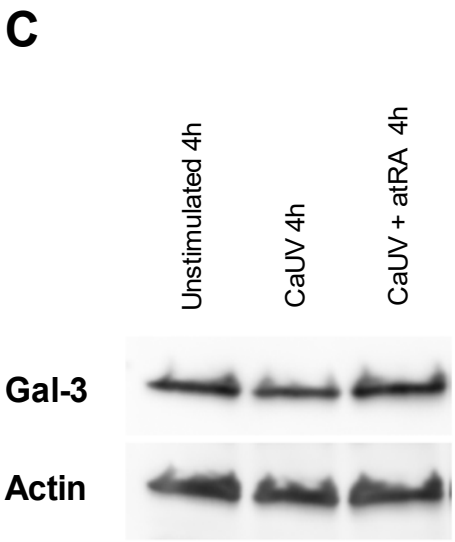

Fig. 16C: Results of Gal-3 are shown in a western blot plot. Actin was used as a control.

Fig. 17: Modulatory effect of atRA on the cytokine expression of TNFα, IL6 and IL12b after different periods of pre-treatment in fungal infection

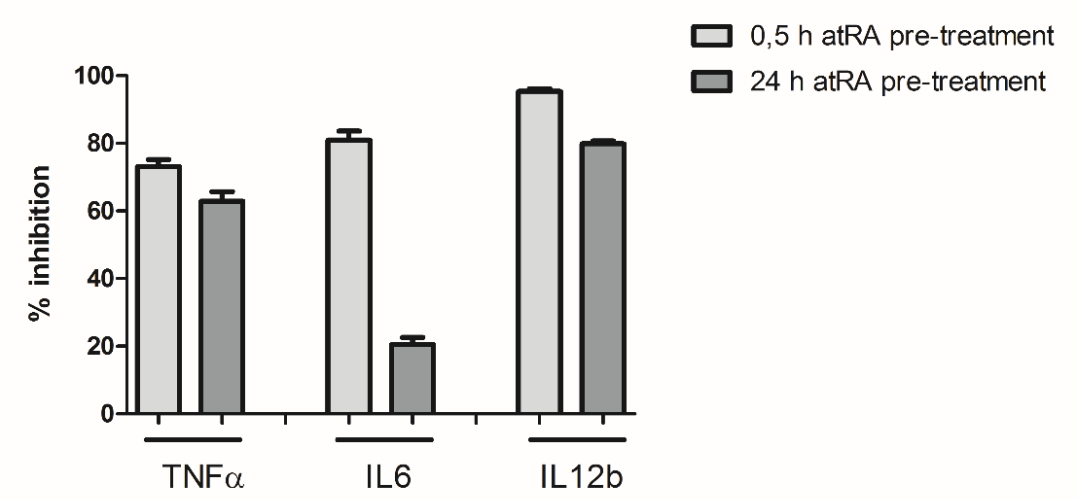

Fig. 17: Monocytes were pre-stimulated with atRA for either 0.5 hours or 24 hours before challenged with UV-inactivated *C. albicans* yeast for 5 hours. The figure shows the inhibitory impact of atRA on the expression of the pro-inflammatory cytokines TNFα, IL12b and IL6 in percent +/- SEM of three independent experiments.

4.4 AtRA exerts its anti-inflammatory effect in a RAR-dependent manner

4.4.1 RAR expression pattern in human monocytes

AtRA exerts most of its biological function by binding one of the RARs. Therefore, we investigated if the effect of atRA is RAR-dependent. First, we investigated the expression pattern of all known RARs in unstimulated monocytes. Monocytes of all five healthy donors showed an expression of both RARα and RARγ, whereas no expression of RARβ was observed (Fig 18A).

4.4.2 Agonist/ antagonist experiments

After determining the expression profile of retinoic acid receptors in monocytes, it remained unclear whether one of these two expressed retinoic acid receptors is responsible for the atRA -mediated modulation on the immune response against *C. albicans*. Therefore, we investigated the gene expression of the pro-inflammatory cytokines TNFα, IL6, IL12b and the PRR Dectin-1 in monocytes stimulated with either specific RARα- or RARγ-Agonists (Fig. 18B).

Both agonists were able to resemble the effect of atRA in a significant manner for TNFα, IL12b and Dectin-1. For IL6, only the RARγ-agonist was able to lead to a significant

downregulation of this cytokine (Fig. 18B). Moreover, we next used specific antagonists to confirm that the *C. albicans*-mediated activation of gene expression could be restored in their presence. The antagonists of both RARs restored the *C. albicans*-induced cytokine expression, at least in part in a significant manner. Nevertheless, the expression level of Dectin-1 was not altered by the addition of any of the RAR-antagonists, suggesting an additional RAR-independent mechanism in the atRA -mediated anti-inflammatory immune response. Overall, these data suggest an involvement of both receptors in the modulatory role of atRA in the *C. albicans*-induced immune response.

Fig. 18: Expression profile of retinoic acid receptors in unstimulated human monocytes, and analysis of their involvement in the atRA-mediated modulation of the immune response against *C. albicans*.

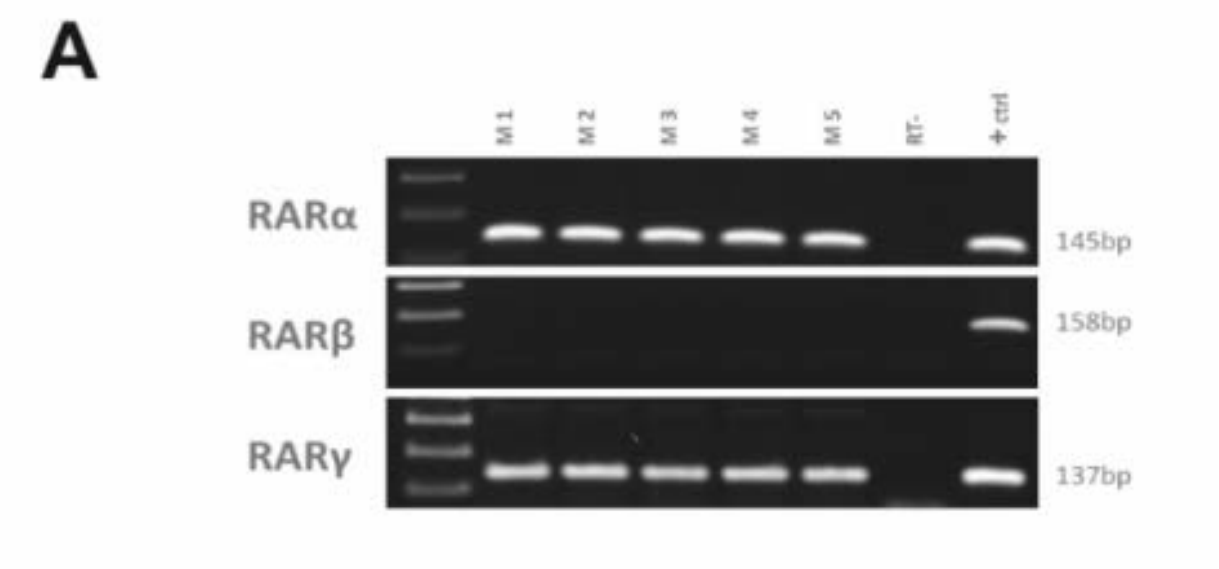

Fig. 18A: The expression profile of RARs was analysed by agarose gel electrophoresis of PCR products from five different donors (M1-M5). PBMCs were used as positive control for the expression of RARα and RARγ, whereas NHBE cells were used as positive control for RARβ. RT-: reverse transcription negative control

Fig. 18B: Analysis of the involvement of the retinoic acid receptors in human monocytes in the atRA-mediated modulation of the immune response against *C. albicans.*

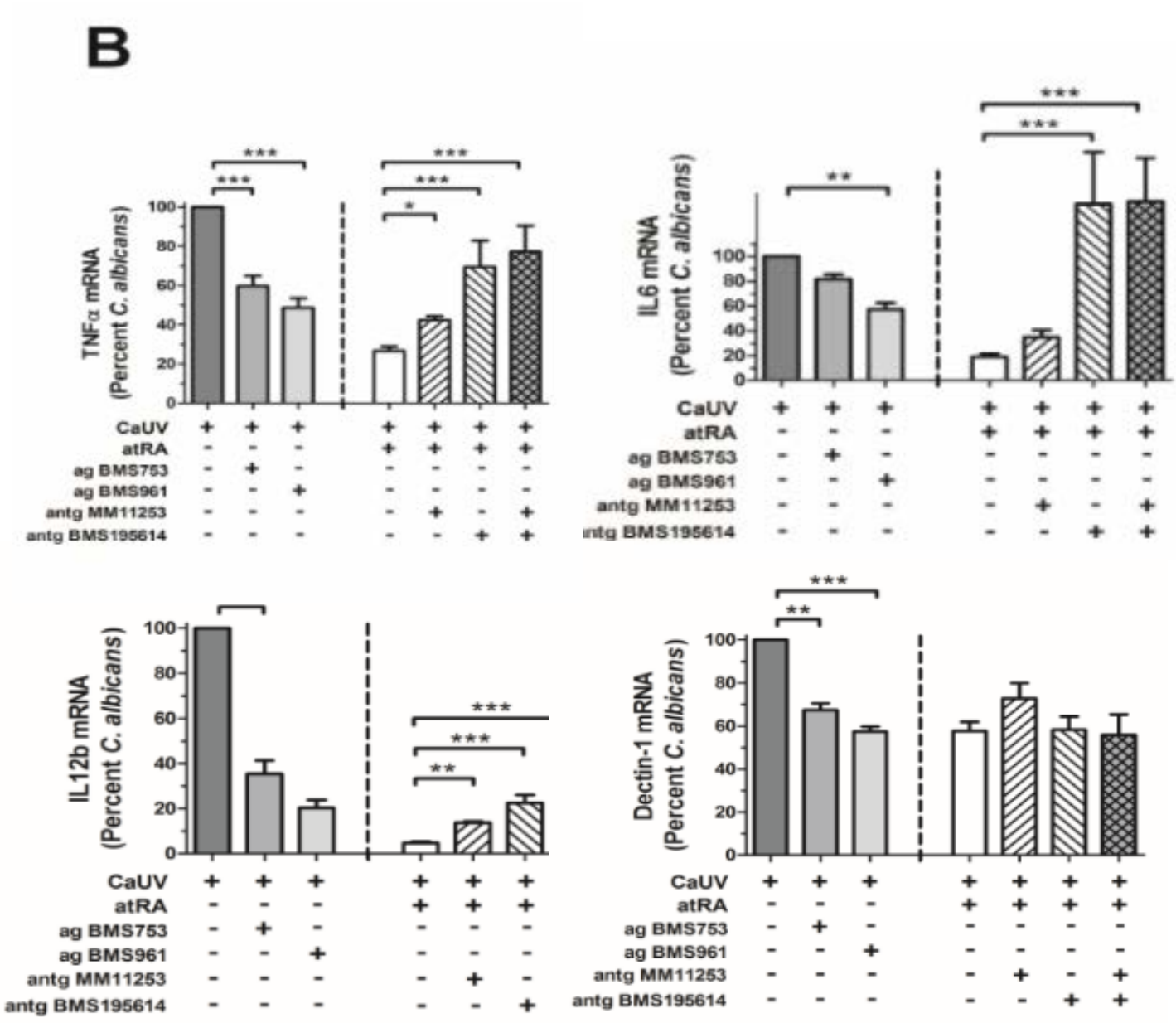

Fig. 18B: Specific RAR agonists (RARα: BMS753; RARγ: BMS 961) and antagonists (RARα: BMS 195614; RARγ: MM11253) were used to address the specific involvement of each RAR on the atRA -mediated downregulation of TNFα, IL6, IL12b and Dectin-1. Data are shown as mean values +/- SEM, assessed from five independent experiments. For statistical analysis, One-Way ANOVA and Dunnett's Multiple Comparison Test were used.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$

4.5 Effect of atRA on the IFN type I response against *C. albicans*

4.5.1 AtRA modulates the IFN-β expression upon *C. albicans* and β-glucan challenge

After investigating the effect of atRA on the classical cytokine response in fungal infections, we next investigated its potential impact on the IFN type I response. In our laboratory, Bräuer already reported the capability of atRA to reduce the *C. albicans*-induced IFN-β production in human monocytes (Bräuer, 2013). We replicated these data. Moreover, we analysed the impact of atRA on the IFN-β expression upon β-1.3 glucan stimulation.

Monocytes challenged with UV-inactivated *C. albicans* yeast for a period of 5 hours, showed a clear up-regulation in IFN-β expression. The addition of 1 μM atRA led to a clear suppression of the *C. albicans*- triggered IFN-β expression (Fig 19A). A similar result was observed when monocytes were challenged with β-1.3 glucan instead of *C. albicans*. Again, we could observe a downregulation of the β-1.3 glucan-induced IFN-β expression (Fig. 19B).

4.5.2 AtRA modulates the expression of several members of the IFN type I pathway

To understand how atRA exerts its function in the IFN type I pathway in monocytes upon *C. albicans* infection, we investigated the influence of atRA on the complete IFN type I pathway at transcriptional level by RT-qPCR (Fig.20).

In this analysis, we included the receptors and the most important regulating factors for IFN type I signalling.

After 5 hours of incubation with UV-inactivated *C. albicans* yeasts, we observed an upregulation of STAT1, STAT2, IRF1, IRF7 and IRF9, whereas the expression of IFNAR1 and IRF5 was not altered after stimulation (Fig. 20). In addition of atRA, we observed a downregulation of the expression of all *C. albicans*-induced genes as well as a downregulation of the expression of IRF5. However, the expression of IFNAR1 was not altered (Fig.20).

In addition, we also investigated the effect of atRA on two additional TLRs which might be involved in the fungal-mediated IFN type I response: TLR7 and TLR9. In our experiments, we observed a significant downregulation of the mRNA expression of TLR7 in the presence of atRA, whereas no alteration on the expression of TLR9 in monocytes challenged with UV-inactivated *C. albicans* yeast occurred (Fig. 21). Interestingly, the presence of *C. albicans* alone already led to a downregulation of both TLRs at

transcriptional level (Fig.21).

Fig. 19: The modulatory effect of atRA on the IFN-β expression in human monocytes upon *C. albicans* (A) and β-1.3 glucan (B) stimulation

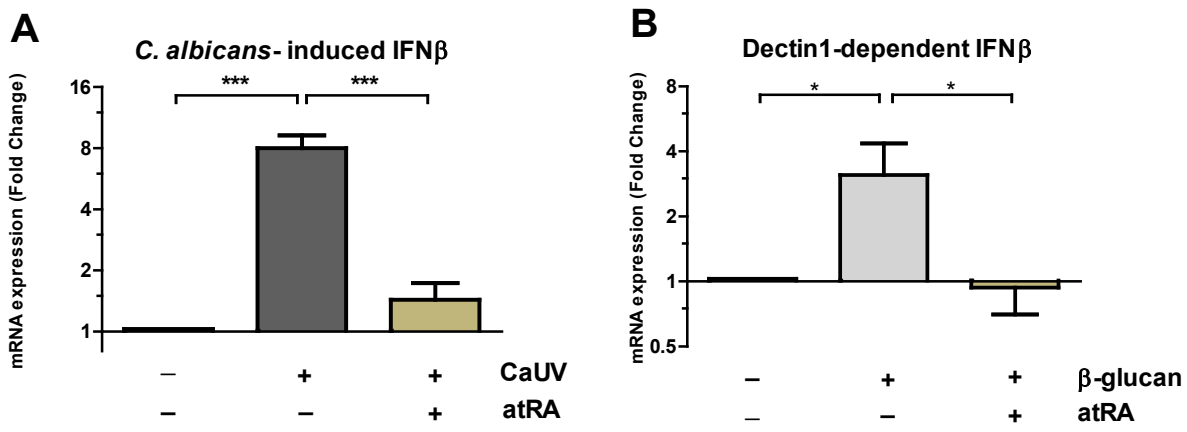

Fig 19A-B: Relative mRNA expression was analysed after 5 hours of incubation with either UV-inactivated *C. albicans* yeast or β-1.3 glucan coated beads in presence or absence of 1 µM atRA. Data are shown as mean mRNA expression +/- SEM of five independent experiments. For statistical analyses, One-Way ANOVA and Dunnett's Multiple Comparison Test were used.
* $p<0.05$; ** $p<0.01$; *** $p< 0.001$

Overall, our data suggest that atRA is able to modulate most of the genes involved in the IFN type I pathway. Moreover, also one of the most important PRRs leading to an IFN type I response (TLR7) was downregulated by atRA.

Next, we addressed whether atRA was capable to modulate the IFN-β induction mediated by these two receptors, TLR7 and TLR9.

Therefore, we stimulated PBMCs for 0.5 hours with or without 1 µM atRA before stimulating for 5 hours with either 5 µg/ ml Imiquimod, a specific ligand of TLR7 or 10 µg/ ml CpG Type A, a synthetic oligonucleotide known to stimulate specifically the TLR9. We observed a clear induction of the IFN-β expression at transcriptional level by the stimulation of the TLR7 and TLR9. Interestingly, the addition of atRA led to a significant downregulation of the TLR7- and TLR9-induced IFN-β response (Fig. 22).

Fig. 20: Immunodulatory role of atRA in the IFN type I pathway upon *C. albicans* infection

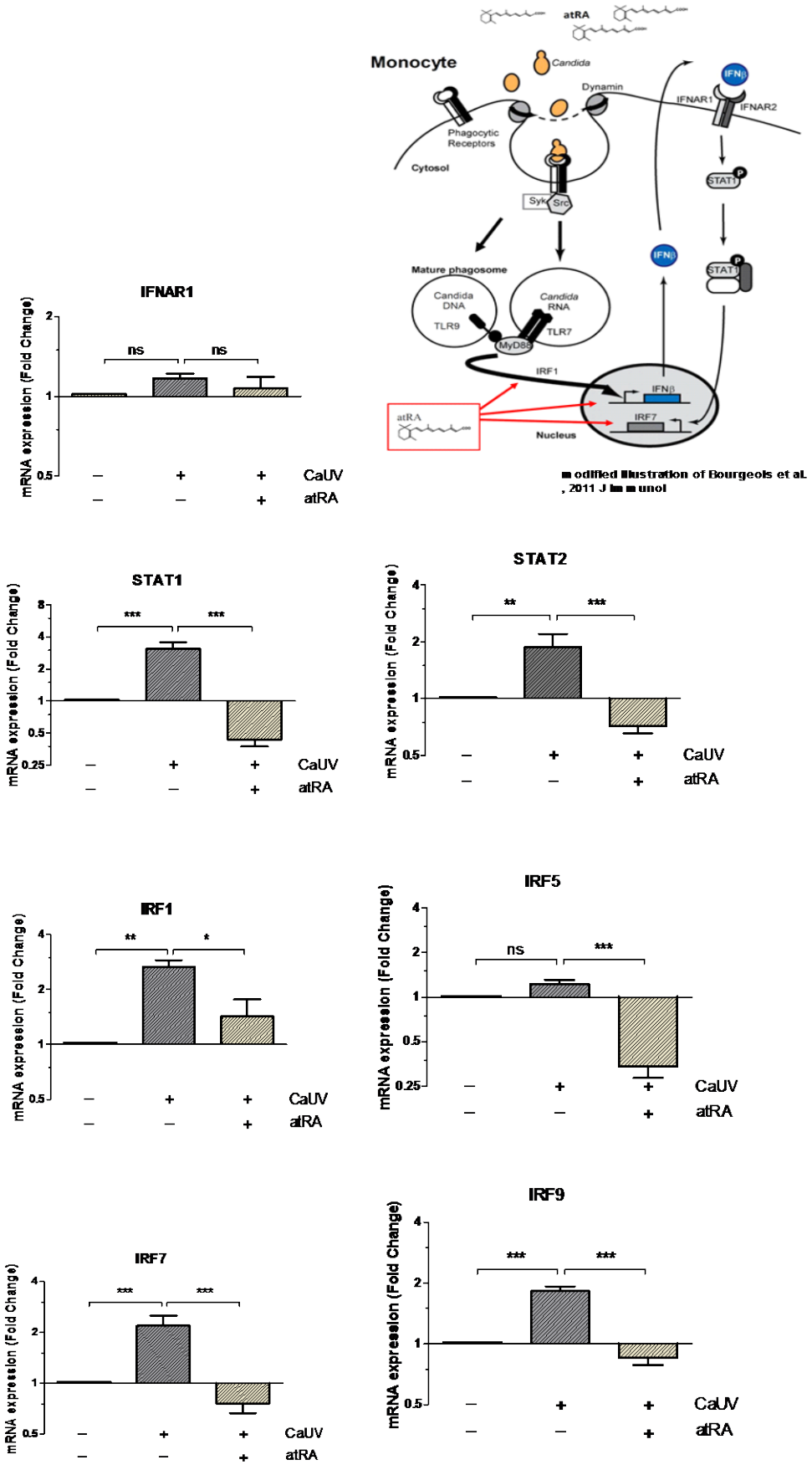

Fig. 20: Expression profile of the most important components of the IFN type I pathway were analysed at transcriptional level by RT-qPCR after monocytes were challenged with *C. albicans* UV- inactivated yeasts for 5 hours in presence or absence of 1µM atRA. Data are shown as mean mRNA expression +/- SEM of five independent experiments. For statistical analyses, One-Way ANOVA and Dunnett's Multiple Comparison Test were used.
* p<0.05; ** p<0.01; *** p<0.001; ns – not significant

Fig.21: Impact of atRA on the expression of TLR7 and TLR9 mRNA upon *C. albican*s infection

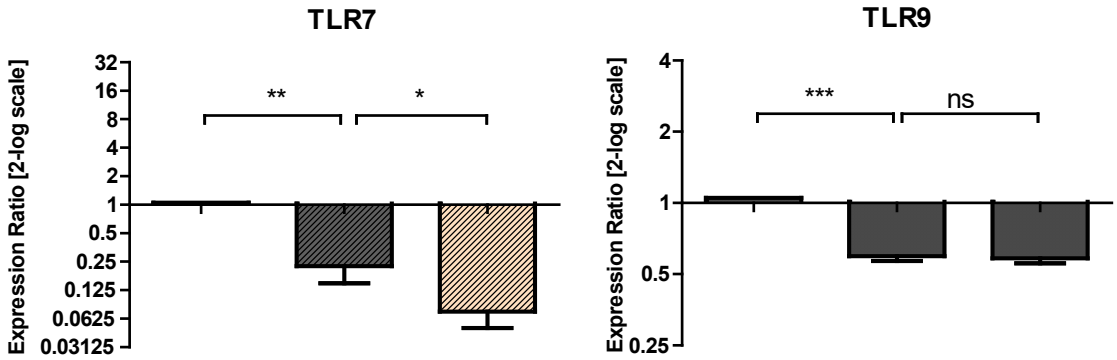

Fig. 21: Expression profile of TLR7 and TLR9 analysed at transcriptional level by RT-qPCR after monocytes were challenged with UV- inactivated *C. albicans* yeast for 5 hours in presence or absence of 1 µM atRA. Data are shown as mean mRNA expression +/- SEM of five independent experiments. * p<0.05; ** p<0.01; *** p<0.001; ns – not significant

Fig. 22: Impact of atRA on the TLR7- and TLR9-mediated IFN-β expression

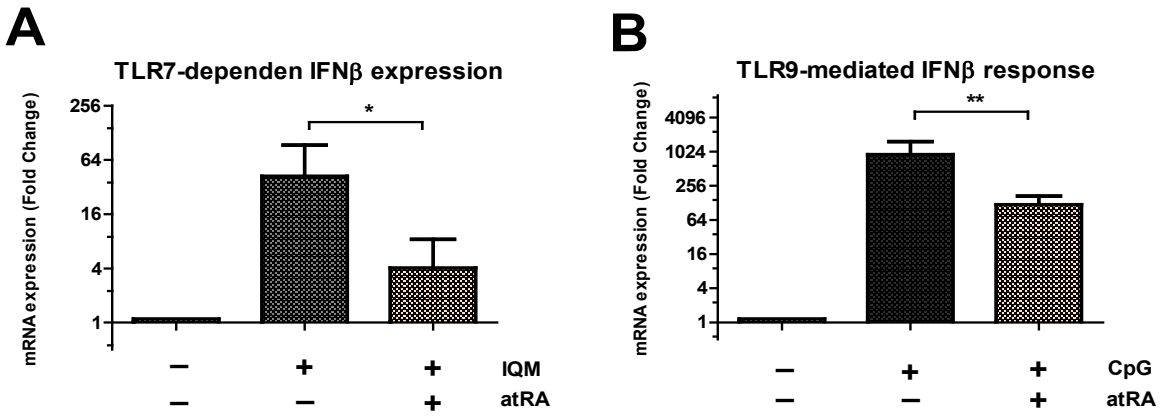

Fig. 22: PBMCs were stimulated for 5 hours with either 10 µg/ ml CpG Type A or 5 µg/ ml Imiquimod in presence or absence of 1 µM atRA. The data are shown as mean mRNA expression +/- SEM of three independent experiments. For statistical analyses, One-Way ANOVA and Dunnett's Multiple Comparison Test were used. * p<0.05; ** p<0.01

5. DISCUSSION

Candida species are one of the most common fungal pathogens of humans and the causative agents of superficial and invasive candidiasis, giving rise to severe morbidity and mortality in millions of individuals worldwide (Naglik, 2014).

Since several centuries, vitamin A deficiency (VAD) has been associated with a reduced immune response affecting the health and survival of infants, young children and pregnant women (West, 2003). Moreover, worldwide VAD is reported to be the third most frequent nutritional problem after protein-energy malnutrition and iron-deficiency and was declared as a public health care problem by the WHO in the years 1990-1993 (Chandra, 1979).

In VAD, especially, viral and bacterial infections are reported to cause high mortality among infants and young children (Ball, 2004, Chandra, 1988, Dollé & Niederreither, 2015, Mayo-Wilson *et al.*, 2011). In fungal infection, nothing is known.

Ribeiro Nogueira *et al*. showed an alteration in retinol in human septic patients treated in ICUs in Brazil (Ribeiro Nogueira *et al.*, 2009). Nevertheless, the role of vitamin A and its mechanism in fungal infections are still unknown.

Our aim was to investigate the function of vitamin A in the *C. albicans*-induced inflammatory response. Furthermore, we wanted to investigate the modulatory role of atRA on the expression and function of Dectin-1 and other relevant PRRs.

We could demonstrate for the first time the modulatory role of atRA in human monocytes in the *C. albicans*-triggered inflammation, modulating the expression of Dectin-1 and its co-receptors as well as regulating the function of Dectin-1.

Furthermore, we investigated the effect of atRA on the *C. albicans*- induced IFN I pathway (Smeekens *et al.*, 2013). We could observe a downregulation of several important regulatory proteins and transcription factors of the IFN I pathway at transcriptional level.

AtRA has a clear immunomodulatory impact upon *C. albicans*- induced immune response in human monocytes.

In our first experiment, monocytes were challenged with UV-inactivated *C. albicans* yeast for 5 hours and 16 hours alone or in the presence of 1 µM atRA. In the presence of atRA metabolite, we observed a clear drop of the *C. albicans*-induced pro-inflammatory cytokines TNFα, IL6 and IL12b at transcriptional and post-translational level measured by real-time qPCR and ELISA.

Since Dectin-1 is one of the most important PRRs in *C. albicans* infection, inducing phagocytosis and complex pro-inflammatory antifungal immune response, we investigated the

role of atRA on the expression of this antifungal PRR and its specific signalling pathway. Therefore, monocytes were incubated with β-1.3 glucan coated beads, a specific Dectin-1 ligand found on the cell wall of *C. albicans* (Brown, 2006, Esteban *et al.*, 2011, Gow *et al.*, 2012).

We observed a significantly increased Dectin-1-mediated cytokine release upon stimulation with β-1.3 glucan coated beads. However, in the presence of atRA, we observed a clear suppression of the β-1.3 glucan-induced pro-inflammatory cytokine production at transcriptional and protein level.

Furthermore, we investigated the effect of atRA on the IL10 production in the same settings. We observed a clear upregulation of the IL10 expression at transcriptional level in the presence of *C. albicans* and while monocytes were stimulated with β-1.3 glucan coated beads. Although we observed no alteration in the IL10 expression in presence of atRA upon *C. albicans* infection as compared to the effect of *C. albicans* alone, we were able to detect an increased IL10 production in the presence of atRA when the cells were challenged with β-1.3 glucan beads.

Wang *et al*. has already shown that vitamin A is able to improve the anti-inflammatory response in LPS- induced infection (Wang *et al.*, 2007). Consequently, we investigated the effect of atRA on the IL10 expression alone. Indeed, we could observe an upregulation of IL10 expression equal to the effect of *C. albicans* alone. We suggest that the IL10 expression induced by *C. albicans* has already reached a saturated state, so that no further additive effect in the presence of atRA can be observed. Overall, atRA seems to modulate the *C. albicans*- and Dectin-1-induced pro-inflammatory cytokine production in an anti-inflammatory manner. Furthermore, atRA seems to increase the anti-inflammatory IL10 cytokine expression until a saturated state.

These data raised the question, whether atRA might regulate the pro-inflammatory cytokine response by modulating the Dectin-1 expression. A recent study reported an ability of vitamin D_3 to modulate the pro-inflammatory cytokine response in

C. albicans infection by the regulation of PRRs (Khoo *et al.*, 2011a). We observed a significant down-regulation of Dectin-1 at transcriptional level after 5 hours and 16 hours in the presence of atRA while monocytes were challenged with *C. albicans*. Interestingly, *C. albicans* alone inhibited the Dectin-1 expression, a mechanism of *C. albicans*, which has not been reported until now, suggesting a new strategy of *C. albicans* to escape the immune response (Bone, 1996, Comstedt *et al.*, 2009).

Moreover, in the presence of atRA we observed a stronger inhibitory impact on the downregulated Dectin-1 expression at transcriptional level and by flow cytometry.

Nevertheless, this inhibitory impact of atRA seems not to be only challenge-dependent, since we observed a similar effect of atRA in the absence of *C. albicans.*

Since vitamin A is known to be important in differentiation processes we investigated if this impact of atRA upon Dectin-1 expression might be due to differentiation of monocytes into dendritic cells (DCs) or macrophages (Chen & Ross, 2004, Mohty *et al.*, 2003). We could observe that also terminally differentiated mature monocytic-derived DCs were sensitive to Dectin-1 modulation by atRA. Comparable to the effect observed on monocytes.

Moreover, we could demonstrate an increasing suppressive effect of atRA on the Dectin-1 expression over a long-term period. We suggest that the inhibitory immunomodulatory impact of atRA might thereby be sustained over a prolonged period of time.

Furthermore, we observed a clear suppressive effect of atRA on the *C. albicans*-induced expression on the Dectin-1 co-receptors TLR2 and Gal-3. These results are in agreement with several reports, investigating an orchestration of several PRR like Dectin-1 and its co-receptors TLR2 and Gal3 leading to an increased efficiency in the anti-*C. albicans* host response (Esteban *et al.*, 2011, Goodridge & Underhill, 2008, Jouault *et al.*, 2006, Linden *et al.*, 2013). These results raised the question, whether the observed atRA-mediated downregulation of Dectin-1 and its co-receptors might be responsible for the drop in the pro-inflammatory cytokine production that we described. Since we observed a downregulation of all *C. albicans*-induced pro-inflammatory cytokines in the presence of atRA after 5 hours of incubation, we investigated the effect of vitamin A on Dectin-1 and its co-receptors at an earlier time point.

Interestingly, we could already observe a downregulation of Dectin-1 on the surface of monocytes after only 4 hours of stimulation, although in a very incipient manner. At this stage, we observed no modulation of the Dectin-1 co-receptors at the post-translational level.

Therefore, we cannot exclude that the atRA-mediated modulation of Dectin-1 might contribute at least in part to the drop of the *C. albicans*-induced cytokine production. Nevertheless, it is likely that other direct mechanisms play a prominent role in the early phase of the atRA-mediated immunomodulation. This is supported by our observation that the short atRA pre-treatment of 0.5 hours led to a stronger inhibitory effect than a long-term pre-treatment of 24 hours, when the atRA-mediated Dectin-1 suppression reached its maximum. This is also in agreement with the observation that atRA modulates the LPS-induced immune response in an anti-inflammatory manner, a Dectin-1-independent immune response, at least in other innate immune cells like macrophages (Hong *et al.*, 2014, Mehta *et al.*, 1994).

In monocytes, so far only Wang *et al.* and Oeth *et al* have investigated the potential immunomodulatory role of atRA on LPS-induced responses (Oeth *et al.*, 1998, Wang *et al.*, 2007).

Whereas Oeth *et al*. did not observe any changes in the LPS-induced TNFα-response in the presence of vitamin A in human monocytes, Wang *et al*. reported only a slight downregulation of the LPS-induced TNFα and IL12 expression (Oeth *et al.*, 1998, Wang *et al.*, 2007). Nevertheless, we observed nearly a 100 % abrogation of the Dectin-1-mediated expression and secretion of TNFα, IL6 and IL12b. It might be interesting to investigate if the mentioned variances in cytokine inhibition might be related to the nature of immunological challenge. Furthermore, the TLR4/ LPS-signalling pathway differs fundamentally from the Dectin-1 activation (Drummond & Brown, 2011). It might be possible, that different PRR- signalling pathways react with a different sensibility to the atRA modulation.

Further studies are needed to investigate and understand the mechanism of the atRA-mediated modulation of the immune response.

Since atRA mainly exerts its function by binding its NRs, the RARs, we investigated the expression of all known RARs in unstimulated human monocytes. We observed an existent expression of RARα and RARγ, whereas an expression of RARβ was absent. These results are in agreement with earlier studies (Fritsche *et al.*, 2000). We used RAR-specific agonists and antagonists and could observe that both, RARα and RARγ are involved in the atRA-mediated downregulation on the *C. albicans*-induced cytokine production of TNFα, IL6 and IL12b. Nevertheless, the modulatory role of atRA on the Dectin-1-mediated immune response seems mainly to occur in a RAR-independent pathway.

In recent years, investigations on the impact of vitamins on immunity and inflammation increased, especially in viral and bacterial infections (Demetriou *et al.*, 1984, Khoo *et al.*, 2011b, López-Varela *et al.*, 2002, Mangin *et al.*, 2014, Mikirova & Hunninghake, 2014, Semba, 1999). In fungal infections, less is known.

Intense research on vitamin D_3 upon fungal infection arose in the recent years, suggesting vitamin D_3 as a potent immunomodulator in invasive candidiasis, being able to act either as pro- or anti-inflammatory agent in a dose dependent manner (Khoo *et al.*, 2011a, Lim *et al.*, 2015). Supplementation of vitamin A in vitamin A-deficient infants and children showed a reduced mortality in several viral and bacterial infections (Mayo-Wilson *et al.*, 2011, West *et al.*, 1991). Only few studies have investigated the effect of vitamin A in humans or animals suffering from sepsis. Demetriou *et al.* showed an increased protective role in the outcome of septic rats in the presence of retinol (Demetriou *et al.*, 1984). Martire-Greco *et al.* observed the effect of vitamin

A on the LPS-induced post-sepsis immunosuppression in a mice model (Martire-Greco *et al.*, 2014). In the presence of atRA, she observed an increased recovery of the immunocompetence in these immunocompromised mice (Martire-Greco *et al.*, 2014). Furthermore, recent studies have reported a decrease in the circulating concentration of antioxidant components of the host defence system in sepsis, including vitamin A, C, E and β-carotene (Ribeiro Nogueira *et al.*, 2009). Ribeiro Nogueira *et al.* monitored the serum level of retinol and β-carotene in septic patients admitted in ICUs in Brazil (Ribeiro Nogueira *et al.*, 2009). He reported an important inadequacy of retinol and β-carotene in septic patients (Ribeiro Nogueira *et al.*, 2009). He observed a significant lower level of β-carotene and higher level of CRP in septic patient without supplementation of vitamin A (Ribeiro Nogueira *et al.*, 2009). These results might be an important hint for further prophylactic and therapeutic approaches for patients suffering from septic infections. Nevertheless, less is known about the effect and mechanism of vitamin A in fungal infections. Further studies are urgently needed.

Beside the PRR-mediated antifungal host defence, the IFN I pathway has recently been demonstrated to play an important role in the anti-*Candida* host defence (Majer *et al.*, 2012, Smeekens *et al.*, 2013).

The IFN-β response, induced by *C. albicans* either by activating the IFN I receptor or other receptors, like the TLRs or CLRs, initiates a complex antifungal host defence, including infiltration of innate immune cells to the place of inflammation to diminish the fungal pathogen load (Biondo *et al.*, 2011, del Fresno *et al.*, 2013, Smeekens *et al.*, 2013).

Controversial data have been published regarding the involvement of Dectin-1 in the IFN I response. While Bourgeois *et al.* showed that Dectin-1 seems not to be involved in the IFN-β response against *Candida* in DCs, del Fresno *et al*. showed a Dectin-1-dependent IFN-β release in DCs (Biondo *et al.*, 2012, Bourgeois *et al.*, 2011, del Fresno *et al.*, 2013). Our results support the involvement of Dectin-1 in the IFN I response in human monocytes.

The role of vitamin A in the IFN I pathway remains until now still unclear. We investigated the influences of atRA in the expression of IFN-β. We could demonstrate a downregulation of the *C. albicans*- and Dectin-1-mediated IFN-β response in the presence of atRA. In the next step, we investigated the function of atRA on the most important regulating factors in the IFN I signalling. We observed a significant atRA- mediated inhibitory effect on all of the *C. albicans*-upregulated IFN I regulating factors after 5 hours of incubation. Only the expression of the IFN I receptor (IFNAR1/2) was not altered in the presence of either *C. albicans* or *C. albicans* and atRA.

Although we observed a significant atRA-mediated downregulation of the IFN-β expression, we were unable to detect IFN-β in the supernatant. Nevertheless, IFN-β is known for its low expression levels in *vivo* and in *vitro* and thus remains below the detection limit at protein level (Majer *et al.*, 2012). Nonetheless, we could verify a significant downregulation of the *C. albicans* and β-1.3 glucan-induced IFN-β mRNA expression in the presence of atRA.

Besides IFNAR1, other receptors including the TLR7 and TLR9 have been reported to promote an IFN-β response by sensing fungal nucleic acids (Biondo *et al.*, 2012, Bourgeois *et al.*, 2011, He *et al.*, 2013, Kasperkovitz *et al.*, 2011).

The TLR7 has been reported to play an essential part in the IFN-β response in *C. albicans* infection (Bourgeois *et al.*, 2011, Naglik, 2014). The function of TLR9 in fungal infection is still not clear. It seems to be an important receptor for fungal recognition (Kasperkovitz *et al.*, 2011, van de Veerdonk *et al.*, 2008). However, no increased susceptibility has been observed in TLR9 knockout mice (Kasperkovitz *et al.*, 2011, van de Veerdonk *et al.*, 2008). In our study, *C. albicans* inhibited the expression of both TLRs in human monocytes after 5 hours of stimulation, which has not been reported so far and might be another mechanism of *C. albicans* evading the host immune response (Bone, 1996, Comstedt *et al.*, 2009). The presence of atRA lead to an increased downregulation of the TLR7, but did not alter the TLR9 expression. This is in agreement with Bourgeois *et al.*, who reported that the TLR7-signalling promotes a pivotal role in the fungal pathogen recognition and mediates the IFN I signalling induced by a fungal pathogen (Bourgeois *et al.*, 2011).

In the next step, we investigated whether the IFN-β release by these specific TLRs might be modulated in the presence of atRA. For this purpose, we stimulated human PBMCs either with the specific TLR7-agonist, Imiquimod or the specific TLR9- agonist CPG Type A for 5 hours in the presence or absence of 1 µM atRA and measured the IFN-β expression on transcriptional level. The stimulation of each TLR leads to a significant increase in IFN-β mRNA expression. In the presence of atRA, the IFN-β mRNA expression was significantly downregulated. Taken together, our data show a huge impact of atRA on the IFN Type I signalling pathway and the expression of IFNβ.

Although certain expression of IFN Type I has clearly shown to be necessary for an accurate immune response against C. albicans, an increased IFN-β release due to disseminated and invasive candidiasis can lead to a detrimental exaggerated host response (Majer *et al.*, 2012).

In this sense, Majer *et al.* reported a remarkable protection against invasive *C. albicans* infection in mice caused by a reduced recruitment of inflammatory myeloid cells (Majer *et al.*,

2012). This data would support a potential protective effect of atRA on Candida-induced sepsis. Further in vivo studies are needed to confirm this hypothesis.

6. CONCLUSION

In the present work, we have demonstrated for the first time, that atRA plays an important modulatory role on the immune response of human monocytes against *C. albicans*. We observed that atRA is supressing the expression and release of the pro-inflammatory cytokines TNFα, IL6 and IL12b on transcriptional and posttranslational level in human monocytes challenged with *C. albicans.* Furthermore, we showed that atRA is able to modulate the expression of PRRs Dectin-1, TLR2, Gal-3 and TLR7 at transcriptional and posttranslational level, which are important mediators for the antifungal host defence.

AtRA is also able to supress the Dectin-1-mediated pro-inflammatory cytokine expression of TNFα, IL6 and IL12b in human monocytes induced by β-glucan, a frequent PAMP on the cell wall of *C. albicans*. At least in part, this downregulation of the pro-inflammatory cytokine expression mediated by atRA could be related to the early modulation of Dectin-1 expression. Nevertheless, the main mechanism of atRA-mediated modulation of the immune response seems to be by a direct mode of action, since longer pre-incubation time reduced the inhibitory effect of atRA compared to the short pre-stimulation with atRA.

Interestingly, vitamin A modulates another pathway, pivotal for *C. albicans* host defence, the IFN I pathway. We observed anti-inflammatory effect of atRA downregulating of the IFN-β expression as well as the expression of most of the important regulating factors known to be relevant in *C. albicans* infection. Furthermore, we observed a downregulation of the TLR7 gene expression, whereas TLR9 seems not to be altered. Both receptors located in the endosomal compartment recognize fungal nucleic acids after their internalization or phagocytosis, mediating an initiation of the IFN-β release and further IFN-β expression.

All together we have demonstrated an important immunomodulatory role of vitamin A in human monocytes challenged with *C. albicans.*

Further studies will help to understand the role of vitamin A in other cell lines of the innate and adaptive immune system in *vitro*. Furthermore, in *vivo* studies are urgently needed, since in *vitro* data only show a small item of the immune system. New investigations on vitamin A in *C. albicans* infection, realised in animal trials are already taking place in our research group.

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8.1 AFFIDAVIT /EHRENWÖRTLICHE ERKLÄRUNG

Ehrenwörtliche Erklärung

Hiermit erkläre ich, Anja Hanisch, geboren 25. 09.1980 in Merseburg, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist, ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind. Weiterhin erkläre ich, dass mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Frau Prof. H. Slevogt sowie Herr Dr. T. Klassert. Jedoch wurde die Hilfe eines Promotionsberaters nicht in Anspruch genommen und es sind auch keine unmittelbaren oder mittelbar geldwerten Leistungen für diese Arbeit von Dritten zur Verfügung gestellt worden, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
Weiterhin erkläre ich, dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Frankfurt, den 30.03.2017 Anja Hanisch

Ort, Datum

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Was man nicht aufgibt, hat man nie verloren
Zitat von Friedrich Schiller
(1759-1805)

Wenn ihr ein Problem anpackt, wird es euch den Weg zeigen, es zu lösen.
Zitat von Rabindranath Tagore
(1861-1941)